THE INFLUENCE OF CERTAIN METAL SALTS ON BLOOD COAGULATION

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SUMMARY.

A number of metal salts were added to oxalated plasma and blood of man and sheep and their effect on the coagulation process was determined. Four μmoles of Co^{2+} and Mn^{2+} ions had no effect in 2.4 ml. reaction mixture. Inhibition of plasma thromboplastin formation was observed with the ions in the following order: $Hg^{2+} < Zn^{2+} = Cd^{2+} < Fe^{2+} = Fe^{3+} < Cu^{2+}$. Plasma thromboplastin activity was not inhibited by the same concentration of metal salt.

The inhibitory effect of Cu^{2+} could be readily counteracted by ethylene diamine tetra acetate (EDTA), cysteine, serine, histidine and partly by glutamic acid, both in absence and presence of plasma.

The inhibitory effect of Fe^{2+} was counteracted by ethylene diamine tetra acetate and cysteine in the absence of plasma. The inhibitory effect of Fe^{2+} after short incubation with plasma could only be overcome to some extent by EDTA or cysteine. The metal salts were inhibitory in plasma and the plasma euglobulin fraction.

The prothrombin clotting time of plasma determined with human brain suspension was prolonged by the metal salts in the following order $Fe^{2+} < Hg^{2+} < Cu^{2+}$.

The prothrombin clotting time of plasma determined with Russell's viper venom was either not influenced or slightly shortened by similar concentration of Hg^{2+}.

The thrombin clotting time of plasma was shortened by Hg^{2+} and Cu^{2+} but slightly prolonged by Fe^{2+}.

INTRODUCTION.

During an investigation concerned with factors which influence the formation of blood thromboplastin it became desirable to determine the effect of certain metal salts on several phases of the blood coagulation mechanism.

Materials and Methods.

Venous blood of normal human donors and blood either from the carotid artery or from the external jugular vein of normal sheep was used. One volume of 0·1M sodium oxalate

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and nine volumes of blood were mixed. Oxalated plasma was obtained after centrifugation at 2000 g. for 30 minutes at + 4° C. Barium sulphate treated oxalated plasma will be called Ba-plasma. The euglobulin fraction was prepared from oxalated plasma. Details for these procedures and references are given by Fantl and Ward (1957).

Serum was prepared from whole blood collected with glass wool after two hours at 37° C.

A phospholipid preparation (P-lipid) was isolated from human brain according to Fantl (1958).

The thromboplastin generation test was carried out with Ba-plasma and serum, otherwise according to Biggs and Douglas (1953).

Prothrombin clotting times were determined (a) with homologous brain suspension similar to the technique of Quick (1951), and (b) with Russell's viper venom (R.V.V.) and P-lipid in veronal buffer pH 7·2 and 0·025M calcium chloride. Russell's viper venom was obtained from Ross Allen, Florida.

Thrombin was prepared from human plasma and decalcified according to Fantl (1958).

Proteins were determined by the Kjeldahl procedure. The metal salts were of analytical grade (B.D.H.). Disodiumethylene-diamine-tetra-acetate will be called EDTA.

RESULTS.

The influence of metal salts on plasma thromboplastin formation.

To normal oxalated plasma of man or sheep were added P-lipid, calcium chloride, and the metal salts in concentrations indicated in the Tables 1, 2, and 3. The reaction mixtures were incubated and aliquots were tested for their ability to clot normal homologous oxalated plasma in the presence of calcium chloride. Results are shown in Table 1.

Results of experiments carried out with $FeSO_4$ are given in Table 2 which includes also thromboplastin generation tests.

Results of experiments carried out with $CuSO_4$ are given in Table 3.

From the results given in Tables 1, 2 and 3 it is seen that although there is some scatter of the observed coagulation times the metal salts can be grouped with regard to their influence on coagulation in the following order. Expressed as μmoles per 2·4 ml. reaction mixture, Co^{2+} and Mn^{2+} ions showed no effect at 4·0; the following ions were inhibitory: Hg^{2+} between 3·7-4·0, Zn^{2+} between 0·4 and 0·8, Cd^{2+} between 0·7 and 1·1, Fe^{2+} between 0·15 and 0·55, Fe^{3+} between 0·4 and 0·8, and Cu^{2+} at 0·2. The experiments in which Ba-plasma and serum was used instead of oxalated plasma showed that the inhibitory effect of the metal salts was of the same degree in both series. It is therefore apparent that the metal salts inhibit either thromboplastin formation or thromboplastin action.

In further experiments the influence of metal salts on thromboplastin activity was tested. This was carried out with aliquots of reaction mixtures in the absence of the inhibitory metal salts and when shortest clotting time on normal recalcified plasma was obtained, parallel tests were carried out with further addition of metal salts. These results are given in Table 4.

TABLE 1.

Influence of metal salts on plasma coagulation (shortest clotting time is recorded in seconds).

Reaction carried out with:	Metal salt in μmoles										
	Co^{2+}, 4·0	Mn^{2+},4·0	Hg^{2+}				Cd^{2+}		Zn^{2+}		Na^{+}
			3·7	3·7	4·0	4·0	0·74	1·1	0·37	0·74	
	pH		pH				pH		pH		pH
	7·2	7·2	6·8	7·4	6·8	7·4	7·2	7·2	7·2	7·2	6·6—7·9
Human plasma	—	10	—	14·5	>60	17	12	55	14·5	66	9—11
Sheep plasma	10·5	—	50	13—60	15	15	—	—	30	10·5	9—11

The reaction mixture consisted of: 0·12 ml. oxalated plasma, 0·18 mg. P-lipid suspended in 0·6 ml. veronal buffer at the pH shown in the table, 0·6 ml. $CaCl_2$ 0·025M. The metal salts were $CoCl_2.6H_2O$, $MnSO_4.4H_2O$, $HgCl_2$, $3(CdSO_4).8H_2O$, $ZnSO_4.7H_2O$, dissolved in 0·145M NaCl and the volumes were made up with 0·145M NaCl to 2·4 ml. Incubation at 28° C.

0·2 ml. aliquot was tested with 0·2 ml. 0·025M $CaCl_2$ on 0·2 ml. homologous oxalated plasma.

The clotting times were determined until three successive results were identical; or incubation was carried out for at least 20 minutes. The controls gave shortest clotting times in 2-5 minutes.

TABLE 2.

Influence of iron salts on plasma coagulation (shortest clotting time is recorded in seconds).

Reaction carried out with:	Addition of $FeSO_4.7H_2O$										
	μmoles	0·185		0·37			0·55				0
	pH:	6·6	7·4	6·6	7·0	7·4	6·3	6·6	7·0	7·4	6·3—7·4
Human plasma		—	20	>60	—	30 (19-60)	>60	44	22	23	9
Sheep plasma		27	20 (16-27)	20 (12-32) 20*-25*	19	24 (17-38)	45	45-58	22	28 (18-50) 24*	11
Human Ba-plasma and serum		21	—	—	25	—	—	>60	—	—	9-11
Sheep Ba-plasma and serum		—	—	—	12	20-23	—	26-50	28	—	9-11

In the case of the plasma experiments, the reaction mixtures were prepared similarly to those given in table 1.

In cases where Ba-plasma and serum was used the reaction mixtures contained 0·12 ml. Ba-plasma and 0·06 ml. serum. Other conditions similar to those in table 1.

Clotting times marked * were obtained with Fe^{3+}. $FeNH_4(SO_4)_2.12H_2O$ was used.

Results outside the brackets are the average clotting times.

TABLE 3.

Influence of $CuSO_4$ on plasma coagulation (shortest clotting time is recorded in seconds).

Reaction carried out with:	Addition of $CuSO_4.5H_2O$ μmoles			
	0·185	0·245	0·37	0·55
Human plasma	35	20-26	50->60	—
Sheep plasma	13·5	15-43	(26-52) 36	40-50
Human Ba-plasma and serum	—	—	33	—

The reaction mixtures were prepared similarly to those given in tables 1 and 2. The pH was 7·4

TABLE 4.

Influence of metal salts on plasma thromboplastin activity (clotting time is recorded in seconds).

Plasma thromboplastin activity determined with:	Activity tested with: $CaCl_2$+metal salt in μmoles					
	Cu^{2+}+0·26	0·37	Zn^{2+}+0·3	Fe^{2+}+0·15	0·37	Na^+
Human plasma	—	—	11	—	—	13
Human Ba-plasma and serum	—	14-15	—	—	12·5-15·5	14
Sheep plasma	—	—	8	—	—	9
Sheep plasma	10	—	—	12-13	—	10-12

The reaction mixtures had the same compositions as those given in table 1 and table 2, pH 7·0–7·3.

The results in Table 4 show that Cu^{2+}, Zn^{2+} or Fe^{2+} which inhibited coagulation in experiments given in Tables 2 and 3, had practically no influence on thromboplastin activity.

It was of interest to find whether the inhibitory effect of the metal salts could be counteracted by chelating agents. Representative experiments are recorded in Table 5.

It can be seen from the results in Table 5 that the inhibitory effect of Cu^{2+} was counteracted by EDTA, cysteine, serine and histidine and partly by glutamic acid, but the inhibitory effect of Fe^{2+} was partly counteracted by EDTA and cysteine, but this was not uniform. Therefore, experiments were carried out to find the best conditions for binding of Fe^{2+} and Fe^{3+} by EDTA. Oxalated plasma was incubated with either Fe^{2+}, Fe^{3+} or Cu^{2+} and EDTA was added after varying incubation times. These results are shown in Table 6.

TABLE 5.

Influence of metal salts and complexing agents on plasma coagulation (shortest clotting time is recorded in seconds). Complexing agents in μmoles.

Metal salts in μmoles	EDTA	Cysteine	Glutamic Acid	Serine	Histidine
Cu^{2+} (0·24) 19-50	(0·54) 13	(3·3) 9-11	(5·3) 12-15	–	(1·5) 9·5
Cu^{2+} (0·38) 30, 50, >60	(0·27) 14	(3·4) 11	–	(9·5) 9-10	–
Cu^{2+} (0·54) 50-60	(0·8) 10·5	–	–	–	–
Zn^{2+} (1·1) 50	(0·8) 13·5	–	–	–	–
Cd^{2+} (1·1) >60	(0·8) 11	–	–	–	–
Fe^{2+} (0·185) 20	(0·135) 20	–	–	–	–
Fe^{2+} (0·192) 19	–	(3·4) 14	–	–	–
Fe^{2+} (0·22) 18-25	–	(3·3) 10-15	(5·3) 19	–	(1·5) 17
Fe^{2+} (0·3) 25	–	(3·4) 15	–	–	–
Fe^{2+} (0·32) 20-29	–	–	–	(9·5) 20	–
Fe^{2+} (0·37) 21	–	–	–	(9·5) 21-30	–
Fe^{2+} (0·48) 50	–	–	–	(9·5) 30	–
Fe^{2+} (0·55) 22, 50, 84	(0·8) 13-20	–	–	(9·5) 22	–
Fe^{2+} (0·64) 60	–	–	–	(9·5) 35	–
Fe^{2+} (0·58) 24	(0·8) 13	–	–	–	–
Na^{+} 9-11	(0·8) 9-11	(3·3) 9-10	(5·3) 10-11	(9·5) 9-11	(1·5) 9

The reaction mixtures consisted of 0·12 ml. oxalated human or sheep plasma, otherwise similar to those in table 1. The quantities added are given in brackets.

TABLE 6.

Influence of plasma on chelation of iron and copper sulphate with EDTA.

Metal salt and EDTA in μmoles	Clotting time in seconds
Cu^{2+} 0·37	26-54
Cu^{2+} 0·37+EDTA 0·54 incubated in presence of plasma	10
Cu^{2+} 0·74	50
Cu^{2+} 0·74 incubated with plasma for 4 mins. + EDTA 0·8	10·5
Cu^{2+} 0·37 + EDTA 0·8 incubated for 1 min. + plasma	10·5
Fe^{2+} 0·37	18-20
Fe^{2+} 0·37 + EDTA 0·54 incubated in presence of plasma. Fe^{2+} 0·37 incubated with plasma for 4 min. + EDTA 0·8	13-20 22-26
Fe^{2+} 0·37+EDTA 0·8 incubated for 1 min.+ plasma	8-10
Fe^{3+} 0·37	18-25
Fe^{3+} 0·37+EDTA 0·54 incubated in presence of plasma	18-20
Fe^{3+} 0·37 incubated with plasma for 4 min.+ EDTA 0·8	20
Fe^{3+} 0·37+EDTA 0·8 incubated for 1 min.+ plasma	9
Na^{+}	9
EDTA 0·8	9

Either oxalated human or sheep plasma was used; pH 7·4, other conditions similar to those in Table 1.

It is seen from the results in Table 6 that Cu^{2+} or Fe^{2+} complexes with EDTA prepared in the absence of oxalated plasma, were not inhibiting plasma coagulation. Inhibition resulting from incubation of Cu^{2+} with oxalated plasma could be overcome with EDTA. In contrast the inhibiting effect of Fe^{2+} or Fe^{3+} produced by incubation with plasma for four minutes was not reversible even with an excess of EDTA.

The cations combine with albumin as well as with certain globulin fractions. The clotting factors are present in the euglobulin fraction. Therefore, a euglobulin fraction of plasma was prepared and the inhibitory effect of metal salts on the euglobulin fraction was compared with that on whole plasma.

Smaller quantities of the inhibitory metal salts are active in the euglobulin fraction than in whole plasma. Again Hg^{2+} showed considerably weaker inhibition than Cu^{2+} or Fe^{2+}. Further, the phenomenon that Cu^{2+} could readily be chelated by EDTA and Fe^{2+} only when chelated in absence of plasma proteins is in agreement with the earlier plasma experiments. A comparison between the activity of Cu^{2+} and Fe^{2+} on plasma as well as on whole blood was carried out and the results shown in Table 7 were obtained.

TABLE 7.

Influence of metal salts on coagulation of whole blood.

Metal salt used μmoles	Whole blood*		Plasma†	
	Inc. time (mins.)	Clotting time (secs.)	Inc. time (mins.)	Clotting time (secs.)
Cu^{2+} 0·96	20	49	—	—
Cu^{2+} 0·384	—	—	9	50
Fe^{2+} 0·97	26	21	—	—
Fe^{2+} 0·194	—	—	12	19
Na^{+}	8	17	3	12

* 0·25 ml. oxalated sheep's blood was used. 35×10^4 platelets/mm^3 were present. P-lipids were not added. Other conditions similar to those in table 1.

† 0·12 ml. oxalated sheep's plasma was used. Other conditions similar to those in table 1.

It is seen from the results in Table 7 that in order to obtain a similar degree of inhibition approximately 2·5-5 times more metal salt is required for whole blood than for plasma. This difference is probably due to the uptake of metal ions by the erythrocytes.

The influence of metal salts on prothrombin clotting time.

The influence of metal salts on the prothrombin clotting time was investigated with plasma in the presence of brain extract. Results are shown in Table 8.

It is seen from the results in Table 8 that the metal salts in an amount of 0·37 μmoles prolonged the prothrombin clotting time. Comparing equimolecular amounts, Cu^{2+} was a more potent inhibitor than Hg^{2+}, and Fe^{2+} had the

TABLE 8.

Influence of metal salts on prothrombin time in presence of homologous brain extract (clotting time in seconds).

Metal salts in μmoles	Human brain extract		Sheep brain extract 3 p.c.
	6 p.c.	0·6 p.c.	
Hg^{2+} 0·74	—	—	29
Hg^{2+} 0·37	21-25	35-36	15
Cu^{2+} 0·37	25-38	46-103	30-42
Fe^{2+} 0·74	—	—	15-19
Fe^{2+} 0·37	20-23	31-35	15·5
Na^{+}	15-19	23-25	12-14

To 0·1 ml. oxalated human or sheep plasma was added first 0.1 ml. of the metal salt dissolved in 0·145M NaCl and then 0·2 ml. of a mixture consisting of equal volumes of the homologous brain suspension and 0·025M or 0·01M calcium chloride. Temperature 37° C.

TABLE 9.

Influence of metal salts on prothrombin time in presence of Russell's viper venom. (Clotting time in seconds).

Metal salt in μmoles	Human plasma P-lipid added										Sheep plasma P-lipid added			
	0 immed.			0	2 min. inc.	30 μg immed.			30 μg 2 min. inc.		0 immed.	0 2 min.inc	30 μg immed.	30 μg 2 min.inc
Hg^{2+} 0·74	—	—	—	—	—	—	—	—	—	—	25	33	7	7
Hg^{2+} 0·44	—	—	—	—	—	—	11	—	—	—	—	—	—	—
Hg^{2+} 0·37	17	—	22	15	—	7·5	—	8	8	8	—	—	—	—
Cu^{2+} 0·37	>60	80	>180	>60	110	13·5	16·5	14	13·5	15	>60	—	11	11
Cu^{2+} 0·185	20	—	—	21	—	—	—	—	—	—	33	33	—	—
Fe^{3+} 0·74	—	—	—	—	—	—	—	—	—	—	>60	—	13	16
Fe^{3+} 0·37	40	69	89	>60	82	12	14	13	13·5	22	57	>60	—	—
Fe^{3+} 0·185	20	—	—	56	—	—	—	—	—	—	—	—	—	—
Na^{+}	19	37·5	38·5	19	28	8·5	12	9	7	8	33	30	7	6

0·1 ml. oxalated human plasma or sheep plasma was used to which was added 0·1 ml. of the metal salt dissolved in 0·145M NaCl; in case of P-lipid addition this was suspended in 0·1 ml. veronal buffer pH 7·2; 0·05 ml. of R.V.V. dissolved in veronal buffer 7·2 was added. This contained 1-2 μg. dried venom. In the experiment under "immed." 0·1 ml. 0·025M $CaCl_2$ was added immediately afterwards. In the experiment under "2 min. inc." the reaction mixture was incubated for 2 min. at 37° C. before $CaCl_2$ was added. Each column represents a different experiment.

least effect. It will be noted this is different from the results of experiments where plasma thromboplastin activity was determined (Table 4). Parallel with the experiments in Table 8 prothrombin clotting times were determined with Russell's viper venom. These results are given in Table 9.

It is interesting to note that whereas Hg^{2+} in an amount of 0·37 μmoles prolonged the clotting time in the presence of brain extract, the same concentration of Hg^{2+} had no effect in the presence of Russell's viper venom. This is similar to the results in Tables 1, 2 and 3, where it was shown that Hg^{2+} had considerably less effect on thromboplastin formation than Cu^{2+} or Fe^{2+}.

The influence of metal salts on the thrombin clotting time.

The time interval between addition of thrombin to oxalated plasma of man and sheep and the appearance of a fibrin gel was determined in several experimental conditions as indicated in Table 10.

TABLE 10.

Influence of metal salts on thrombin clotting time (seconds).

	Human plasma $CaCl_2$ addition		Sheep plasma $CaCl_2$ addition	
Metal salt in μmoles	0	0·1 ml. 0·025M	0	0·1 ml. 0·025M
Hg^{2+} 0·37	12	—	8	—
Cu^{2+} 0·37	11	—	9	—
Fe^{2+} 0·37	23	13	20	10·5
Na^{+}	18·5	11·5	18	10

0·1 ml. oxalated human or sheep plasma was used to which was added 0·1 ml. of the metal salt dissolved in 0·145M NaCl and either 0·1 ml. 0·075M NaCl or 0·1 ml. 0·025M $CaCl_2$ and finally 0·1 ml. human thrombin free of Ca^{2+} in 0·145M NaCl. Temperature 37° C.

From the results given in Table 10 it is seen that 0·37 μmoles of Hg^{2+} or Cu^{2+} ions reduce the thrombin clotting time very markedly in both human and sheep oxalated plasma, as compared to the control. Fe^{2+} ions are slightly inhibiting. It will be noticed that the acceleration produced by Hg^{2+} or Cu^{2+} is of the same order as on addition of Ca^{2+} ions. However, it is unlikely that Hg^{2+} or Cu^{2+} acts in the same way as Ca^{2+}. The latter reduces the thrombin clotting time by precipitation of the inhibitory oxalate. But neither Hg^{2+} or Cu^{2+} produced insoluble oxalates, and complexes of Hg^{2+} or Cu^{2+} with oxalate are probably not responsible for the observed phenomenon.

It is likely that the metal ions which shortened the thrombin clotting time have a direct effect on fibrinogen, and share this property with a number of unrelated substances (Gerber and Blanchard, 1945; Fantl and Ebbels, 1953).

DISCUSSION.

Frouin and Mercier (1913) found that addition of the salts of rare earths (Ce, La, Nd, Pr, Sm, Th, Y) to dog's or rabbit's blood made it incoagulable. Heard (1917) found that 0·01M Co^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} delayed coagulation

time of blood but no influence was noted at 0·001M. Haüsler and Schnetz (1935) found that 0·0005M of Cu^{2+}, Zn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} inhibited the clotting time of rabbit's blood. Vincke and Schmidt (1942) and Dyckerhoff and Grünewald (1943) investigated the changes in blood coagulation after injection of rare earths into rabbits. They observed an inhibitory effect on the first phase of blood coagulation and on prothrombin formation.

The present experiments show that Hg^{2+} is a weak inhibitor of plasma thromboplastin formation when compared with Cu^{2+}, Cd^{2+}, Zn^{2+}, Fe^{2+} or Fe^{3+}. In view of the great affinity of Hg^{2+} for SH-groups (Gurd and Wilcox, 1956; Barnard and Stein, 1958) this would indicate that SH-groups are either not present in any of the components of the thromboplastin complex or that they are not essential for the coagulation process. The inhibition produced by Cu^{2+}, Cd^{2+}, Zn^{2+} ions suggests that these ions react with essential groups in one or more of the clotting factors of the thromboplastin complex. The inhibitory effect of these ions can be counteracted by EDTA, cysteine, histidine, serine and glutamic acid. It is therefore not possible to locate the groups with which the metal ions react, but from the fact that the inhibitory effect can be counteracted it can be concluded that no denaturation by the metal salts of clotting factors has taken place.

The iron salts occupy a special place among the inhibitory metal salts. First it should be pointed out that although Fe^{2+} ions were added to plasma it is very likely that Fe^{3+} compounds were formed because according to Ehrenberg and Laurell (1955) under aerobic conditions Fe^{3+} transferrin is formed more rapidly with Fe^{2+} than with Fe^{3+} ions. The activity of Fe^{2+} was of the same order as that of Fe^{3+}. It is more convenient to use Fe^{2+} solutions because of the greater hydrolytic stability of Fe^{2+} than Fe^{3+} at the pH range used in the present experiments. Apparently iron is quite strongly bound by plasma components since in contrast to Cu^{2+} the inhibitory effect could not be readily reversed by EDTA. This is similar to the behaviour of Cu^{2+} and Fe^{2+} towards the metal combining globulin (transferrin). Surgenor, Koechlin and Strong (1949) have found that the affinity for Fe^{3+} is higher at neutral pH than for Cu^{2+}.

The experiments indicate that the metal salts inhibit plasma thromboplastin formation but do not influence plasma thromboplastin action in similar concentration. This is supported by experiments with isolated clotting factors in the euglobulin fraction. There is some quantitative difference between plasma and euglobulin fraction. A lower concentration of metal salt was inhibitory in the euglobulin fraction than in whole plasma. This is due to the fact that both albumin and globulins can bind metal ions, and albumin was absent from the euglobulin fraction. However, the order of activity was the same in the different preparations. The metal combining protein can bind 1·25 μg. of iron per mg. of protein (Surgenor *et al.*, 1949). The volume of euglobulin fraction used in the test contained 1·12 mg. protein and coagulation was inhibited by 10·35 μg. Fe^{2+}. It would, therefore, appear that other metal-combining components of

plasma or serum are involved in the inhibition of blood coagulation. It is possible that a protein which can combine with Ca^{2+} ions is essential for the coagulation process and this can be blocked by Cu^{2+} or Fe^{3+}.

The metal salts do not react uniformly in the different phases of the coagulation process. Whereas Hg^{2+} is a weak inhibitor of blood thromboplastin formation it is equally effective as Fe^{2+} as an inhibitor in the prothrombin conversion in presence of brain extract. Sarkar, Chatterjee and Bannerjee (1956) found that 0·5 μmoles Cu^{2+} or Hg^{2+} prolonged the prothrombin clotting time. On the other hand, in the presence of Russell's viper venom a similar concentration of Hg^{2+} was not inhibitory at all but even accelerating. This could be due to the shortening effect of Hg^{2+} on the thrombin clotting time.

A possible explanation for the differing activities is that Hg^{2+} reacts with a plasma or serum component which is not required for blood thromboplastin formation but is required for thromboplastic activity in presence of brain extract.

This component may be factor VII (SPCA, proconvertin). This factor is not involved in blood thromboplastin formation and does not take part in the prothrombin conversion in the presence of Russell's viper venom but factor VII is required for the conversion of prothrombin with brain thromboplastin.

Apart from the above considerations, it could be mentioned that Cu, Zn and Fe compounds occur in tissues and can be present in isolated P-lipids. An inhibitory effect of such products could be due to the metal contamination.

The inhibitory effect of the metal salts on certain phases of the coagulation process may produce bleeding in metal poisoning. Barni, Nucci and Reale (1958) found that rabbits poisoned with cadmium chloride had a prolonged recalcification time of plasma.

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THE EFFECT OF ETHYL TRICHLORACETATE ON LIVER LIPIDS

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SUMMARY.

The results presented indicate that subcutaneous injections of ethyl trichloracetate in appropriate doses will cause a statistically significant fall in liver lipid values and statistically significant increases in liver phospholipids of choline deficient animals.

The extra phospholipids found in the liver had a similar choline content to that found in the choline deficient control livers.

Assessment of the histological changes in such livers does not indicate any toxic effect of ethyl trichloracetate, nor any effect on the changes due to the amino acid imbalance of the diet used. The reduction in liver lipid found by biochemical analysis appears related to a decrease in centrolobular fat.

INTRODUCTION.

It has been reported (Kratzing and Windrum, 1957) that subcutaneous injections of ethyl trichloracetate (ETCA) reduce the content of lipid which accumulates in the liver of choline deficient rats. The present studies extend the earlier investigations and in addition report the effect of administration of ETCA on the phospholipid content of livers from choline deficient rats.

Previous workers have shown that there is a relationship between choline deficiency and the phospholipid content of the liver (Perlman and Chaikoff, 1939; Artom and Cornatzer, 1947; DiLuzio and Zilversmit, 1953).

The present experiments show that rats receiving ETCA have an increased concentration of phospholipid in the liver compared to control choline deficient rats. This increased amount of phospholipid is accompanied by proportionate increases in lipid choline.

MATERIALS AND METHODS.

Animals. Young locally bred white rats of both sexes weighing 50-70 gm. were used. They were housed in small groups in wire cages with wire mesh bottoms. Food and water were provided *ad libitum*.

Diet. The hypolipotropic diet used was deficient in some amino acids and was based on the Series II diet described by Best, Lucas, Ridout and Patterson (1950) and had the following percentage composition:

Casein (British Drug Houses: fat free and vitamin free)—8; gelatin (Davis gelatin)—12; beef dripping—10; peanut oil—2; sucrose—62; Hubbell's salt mixture (Hubbell, Mendel and Wakeman, 1937)—4; vitamin powder—1; vitamin A and D concentrate—1.

The vitamin powder contained thiamine hydrochloride 50 mg.; riboflavin 25 mg.; pyridexine 29 mg.; calcium pantothenate 90 mg.; nicotinamide 100 mg.; folic acid 5 mg.; biotin 5 mg.; vitamin B_{12} 75 μg.; 2 methyl 1:4 naphthoquinone 10 mg.; alpha tocopherol 250 mg.; para amino benzoic acid 1 gm.; inositol 5 gm.; — made up to 100 gm. with fine sucrose.

The vitamin concentrate ("Vetemul", Nicholas (Aust.) Pty. Ltd.) contained 5000 I.U. vitamin A and 500 I.U. vitamin D_3 per 1 gm. When choline supplements were given, they were added at the rate of 0·230 gm. of choline chloride per 100 gm. of diet, which provides 20 mg. of choline per 10 gm. of diet.

Ethyl trichloracetate. The ester was prepared by passing dry HCl gas through a mixture of dry trichloracetic acid and absolute ethanol. After washing and drying the ester, the fraction boiling between 166-168° C. was collected. After storage for some time, the vapour above the liquid ester gave a positive test for phosgene with paper containing dimethylaminobenzaldehyde and diphenylamine. Consequently 2 p.c. ethanol was added to all samples of the ester used for injection, in order to inhibit this phosgene formation.

Treatment. In "mixed" experiments, where animals of both sexes were used, the proportion of males to females was the same in all groups. Animals were fed the diet (with or without choline) for twenty-one to twenty-four days before administration of ETCA was commenced. When the ester was given for only three consecutive days, two subcutaneous injections were given daily. In longer experiments, the rats received once daily subcutaneous injections five days per week. Some animals developed small areas of eroded skin after repeated injections. Control animals did not receive any injections.

Twenty-four hours after the last injection, the animals were killed by stunning and decapitation. The livers were removed, portion taken for histological examination, and the remainder stored at — 20° C. prior to analysis.

Chemical analyses. Liver lipids were extracted by the method of Wheeldon and Collins (1957), the final ethereal solution being evaporated to dryness and the lipid measured gravimetrically. Results were expressed as percentage lipid per wet weight of liver. After the total lipid had been determined it was redissolved in 25 ml. methanol-chloroform (1:2 v/v) and portions taken for the determination of choline and lipid phosphorus.

Choline. 2 ml. portions of the lipid solution were added to glass ampoules and the solvent was removed by means of a stream of N_2. 5 ml. 6N HCl was added to each ampoule and after sealing, the ampoules were kept at 105° C. for 24 hours. On cooling, the hydrolysates were diluted to 50 ml. and choline was determined on aliquots by the method of Kushner (1956).

Phosphorus. Total lipid phosphorus was determined by the method of King (1946) after digestion of the lipids by 0·5 ml. of 70 p.c. $HClO_4$.

Histological method. Portion of the liver was fixed in 10 p.c. formol-saline. Histological sections, prepared in a routine manner, were stained with haematoxylin and eosin. Frozen sections stained with Sudan IV were used to demonstrate fat. The histological changes of centrolobular fat, periportal fat, and hydropic change were given subjective gradings of — to +++ so that different animals and groups could be compared.

RESULTS.

Total lipid content of liver.

The results are summarised in Table 1.

Experiment A showed a significant fall in lipid values with a dosage of 720 μ moles (0·1 ml.) per 100 gm. twice daily for three days. Experiment C, which was a repetition of Experiment A, produced results significant to $P < 0{\cdot}05$. With a smaller dose (0·03 ml. per 100 g.b.d.) a slight fall in the mean liver lipids occurred, but the results were not statistically significant (Experiment B). This experiment also showed that ETCA had no appreciable effect on the liver lipid values of animals receiving a choline supplement to the basal diet.

Experiment D, in which injections were given over a period of one month, showed that repeated injections of ETCA maintained the liver lipids at a low value during a period of continued dietary choline deficiency. Table 4 shows the individual values of the liver lipid content of the injected animals. One animal had a very high liver lipid content which differed greatly from those of the rest of the group, while nine out of the eleven values were below the mean of 7·5.

Experiment E showed that injections of ETCA over a period of one month did not have any appreciable effect on the liver lipid values of animals receiving the basal diet supplemented with choline.

In all these experiments the female rats tended to have somewhat higher liver lipid values than males. However, both sexes showed such variation in response that larger numbers would be necessary for proper evaluation. The results of both males and females have been combined for statistical analysis.

Phospholipid content of liver.

The subcutaneous injections of ETCA produced significant changes in the liver phospholipid content. The results from three experiments are presented in Table 2.

In Experiment B, ETCA administration significantly ($P < 0{\cdot}01$) increased the phospholipid content of livers from animals which received choline supplements. This increase occurred even though the total lipid content of the wet liver was not significantly altered. In choline deficient animals the administration of this small dose of ETCA produced no statistically significant changes in the phospholipid or the total lipid content of the liver. However, the phospholipid did show a tendency to rise and the total lipid a tendency to fall — and these changes are in the same direction as in the statistically significant groups.

However, in Experiment C, where the dosage of ETCA was three times that in Experiment B, the ester did produce a highly significant ($P < 0{\cdot}001$) increase in liver phospholipid content in choline deficient animals. The increase in liver phospholipid content was also highly significant ($P < 0{\cdot}001$) in choline deficient animals, which received ETCA over a four-week period (Experiment D).

TABLE 1.

Effect of ethyl trichloracetate injections on liver lipids.

Experiment	Number of rats	Sex	Mean weight†	Choline*	ETCA dosage μ moles per 100 gm.	ETCA injection per 100 gm.	p.c. liver lipid ‡ mean & S.E.M.	Significance
A	4	Mixed	75·5	0	—	—	18·7±2·3	P<0·02
	4	Mixed	73·8	0	6×720 over 3 days	0·1 ml. b.d.	9·6±1·1	
B	7	Mixed	56·6	0	—	—	18·5±3·0	N.S.
	7	Mixed	56	0	6×220 over 3 days	0·03 ml. b.d.	13·3±2·2	
	7	Mixed	64·5	20	—	—	4·9±0·2	N.S.
	7	Mixed	66	20	6×220 over 3 days	0·03 ml. b.d.	5·3±0·1	
C	12	Mixed	58	0	—	—	10·4±1·1	P<0·05
	12	Mixed	56	0	6×720 over 3 days	0·1 ml. b.d.	7·6±0·5	>0·02
D	13	Female	70	0	—	—	14·2±1·0	P<0·001
	11	Female	70	0	20×1450/26 days	0·2 ml. daily	7·5±1·3	
E	5	Mixed	67	20	20×1450/20 days	0·2 ml. daily	5·0±0·1	

† Mean weight at commencement of injections.
* Choline chloride added. Calculated as mg. choline per 10 gm. food.
‡ Expressed as percentage wet weight of liver.

TABLE 2.

The effect of ethyl trichloracetate on liver phospholipid.

Experiment	Treatment*	Total lipid p.c. wet liver†	Lipid phosphorus		Lipid choline		Ratio lipid choline/lipid phosphorus	
			μ moles/gm. wet liver	μ moles/gm. lipid	μ moles/gm. wet liver	μ moles/gm. lipid	as wet liver	in total lipid
B	choline deficient (7)	18·5±3·0	23·5±1·3	149·3±27·6	10·9±0·6	70·5±14·3	0·46	0·47
	choline deficient +ETCA (7)	13·3±2·2	28·1±2·1	253·9±51·8	13·2±1·2	120·5±27·0	0·47	0·47
	choline deficient+choline (7)	4·9±0·2	28·9±1·1	592·2±24·9	14·9±0·5	305·3±9·3	0·52	0·52
	choline deficient+choline +ETCA (7)	5·3±0·1	35·2±1·2	689·0±35·4	18·0±0·7	341·2±12·0	0·51	0·52
C	choline deficient (12)	10·4±1·1	28·5±1·0	309·0±32·2	13·6±0·8	145·1±17·4	0·48	0·47
	choline deficient+ETCA (12)	7·6±0·5	43·7±1·6	594·5±36·3	19·5±0·9	255·1±19·9	0·45	0·43
D	choline deficient (13)	14·2±1·0	31·1±0·9	230·2±15·5	14·2±0·6	105·6±8·2	0·46	0·46
	choline deficient+ETCA (11)	7·5±1·3	37·5±0·8	584·2±50·1	17·2±0·5	270·8±25·0	0·46	0·48

* Number of animals per group in brackets.
† Mean and S.E.M.

Effect of ETCA on the choline content of phospholipid.

In three experiments analysed (Exps. B, C and D) the administration of ETCA to choline deficient rats produced a rise in total liver phospholipid which was accompanied by proportional increases in amounts of lipid choline. This is seen in Table 2 as the ratio of lipid choline to lipid phosphorus and has been expressed both on a wet liver and total liver lipid basis.

The ratio of choline to phospholipid remains the same in choline deficient animals receiving ETCA as in control choline deficient animals.

On the other hand, the addition of choline to the deficient diet resulted in a slight increase in the choline to phospholipid ratio as well as increasing the amount of total phospholipid.

Histological changes in liver.

In Experiment A the control animals showed hepatic centrolobular accumulation of fat in large and small globules after three weeks on the choline deficient diet. This follows the usual pattern (Hartroft, 1950). The animals receiving ETCA showed no unusual histological features, but the amount of centrolobular fat was much less, and correlated well with the low values obtained by analysis.

In later experiments, the initial weights of the animals were somewhat lower, and these animals showed certain complications in the histological appearances, notably the presence of some periportal fat, a variable degree of hydropic change, and occasionally small areas of cell necrosis. These changes were particularly noticeable in the longer experiments (Exps. D and E) which extended for seven to eight weeks.

Table 3 shows subjective histological gradings given to the changes in the control group of choline deficient animals in Experiment D, and Table 4 shows the values for the group receiving ETCA in Experiment D. The tables show

TABLE 3.

Histological analysis of livers from control choline deficient rats.

Weight* gm.	Liver† lipid	Centrolobular fat	Periportal fat	Hydropic change
60	10·9	++	+	+++
65	17·2	+++	+	++
66	12·2	++	+	++
68	11·2	++	−	+
68	15·5	+++	+	+
78	10·7	++	+	++
76	14·9	++	+	+++
81	22·8	+++	++	−
74	18·6	++	+	−
72	14·1	+++	++	+++
74	10·0	++	+	+
75	12·9	+++	+	++
51	13·7	++	+	++

* Weight at commencement of injection period.

† Expressed as percentage wet weight of liver.

TABLE 4.

Histological analysis of livers from choline deficient rats receiving ETCA.

Weight* gm.	Liver† lipid	Centrolobular fat	Periportal fat	Hydropic change
57	6·2	–	+	–
68	9·6	++	+	++
71	7·1	–	+	+++
72	5·0	–	+	+
73	5·2	–	+	++
79	5·3	–	+	++
75	5·5	+	++	+++
74	5·9	–	+	+
80	6·8	–	+	++
71	5·8	+	+	+++
61	19·9	+++	+	–

* Weight at commencement of injection period.
† Expressed as percentage wet weight of liver.

that periportal fat accumulation was relatively slight in both the control and injected groups. Hydropic change was a variable feature with a considerable range between animals of the same group, but there was no obvious difference between the two groups. By contrast centrolobular fat was greatly decreased in the group receiving ETCA.

In Experiment E the animals received a choline supplement to the basal diet as well as receiving ETCA injections, and their livers showed variable degrees of hydropic change, some periportal fat and occasionally small foci of periportal cell necrosis. These changes could not, therefore, be attributed to choline deficiency.

DISCUSSION.

The word "lipotropic" was introduced (Best, Huntsman and Ridout, 1935) to describe substances which decrease the rate of deposition and accelerate the rate of removal of excess liver fat, and was later extended (Wilgram, Hartroft and Best, 1954) to include the prevention of accumulation of fat in all other tissues (kidney, heart and arterial walls) in which similar phenomena have been demonstrated.

Assessment of the present results leads to the conclusion that ethyl trichloracetate had exerted a lipotropic action in the presence of choline deficiency and had not produced toxic reactions. The animals were in established choline deficiency when the injections were commenced, so that the results show a decrease from expected high values, rather than the maintenance of a low value of liver lipid from the start of a period of choline deficiency. The injections of ETCA did not result in a return to the uniformly low values expected with choline supplements, so that the lipotropic action was only relatively effective. Estimates of relative potency based on such decreases from a variable high level are not useful for the assessment of dose-response curves (Best, Lucas, Ridout and Patterson, 1950).

Large doses of ETCA (of the order of 0·3 ml. per 100 gm. body weight and upwards) proved lethal to rats on both stock and choline deficient diets, but in no case did we find this toxicity to be related to liver changes.

A common pitfall in establishing a lipotropic action has been the failure to appreciate "the pseudolipotropic effect of caloric restriction" (Best, Hartroft and Sellers, 1952) (see also Griffith and Mulford, 1941; Treadwell, Tidwell and Grafa, 1953; and Hartroft, 1954).

In our experiments the animals which received injections for only three days showed no appreciable change in weight which might indicate a reduced food intake. In the longer experiment (Exp. D) the mean weight gain of the injected animals was somewhat less than the mean weight gain of the choline deficient controls. However, there was no correlation between weight gain and liver lipid value in the groups or in the animals individually.

Best and Huntsman (1932) found that subcutaneous injections of saline had some "lipotropic" action when compared to non-injected animals, and stated that frequent handling and subcutaneous injections interfered with deposition of liver fat. Their animals were, however, receiving twenty-four injections each per week for three weeks. It is possible that the giving of an injection of ETCA may, by itself, or because of some tissue destruction at the site of injection, be a contributory factor to the altered lipid metabolism. Other workers have given daily injections and still obtained high values of liver fat in choline deficiency, e.g. Beveridge, Lucas and O'Grady (1945) gave their animals vitamin B supplements by injection.

There has been much interest in the effect of protein on the accumulation and lobular distribution of liver lipids. Best and Huntsman (1935) recognized that dietary protein was a factor affecting the lipotropic action of choline. Beveridge, Lucas and O'Grady (1945) noted the importance of the balance of amino acids, and showed that the lipotropic activity of a protein is determined not only by its content of methionine, but also by the nature and quantity of the other amino acids present. These relationships have been reviewed by Best, Hartroft, Lucas and Ridout (1955) and Harper and Benton (1956). The investigations of these and other authors (e.g., Shils and Stewart, 1954) have led to the development of the general hypothesis that choline lack leads to centrolobular fatty change and that protein inadequacy leads initially to periportal fat accumulation. Though choline lack and centrolobular fat accumulation can be corrected by providing choline precursors such as methionine, choline supplements will not completely prevent the deposition of periportal fat when the protein intake is inadequate. The protein deficiency causes liver cell damage as well as fatty metamorphosis and though large supplements of choline will remove some of this fat, they will not prevent the liver damage (Koch-Weser, de la Huerga and Popper, 1953).

The hypolipotropic diet we used was deficient in essential amino acids (e.g. threonine, tryptophane, phenylalanine and histidine) as well as methionine;

our animals were young and at a stage when growth stimulus would normally be of a high order. It is not surprising, then, that they developed histological evidence of multiple changes in their livers.

Since these changes occurred in the livers of choline supplemented groups, as well as in the choline deficient groups, we have attributed the hydropic degeneration, periportal fat and occasional periportal necroses to the relative inadequacy of essential amino acids, following the general histological descriptions of earlier workers (Wang *et al.*, 1949; Koch-Weser, de la Huerga and Popper, 1953; Nino-Herrera, Harper and Elvehjem, 1954; Shils and Stewart, 1954; Best, Hartroft, Lucas and Ridout, 1955). Though the hydropic degeneration and periportal fat accumulation both appear to be related to the protein inadequacy, they may appear simultaneously or independently of each other. When hydropic degeneration was slight, it occurred only in the periportal areas.

The fact that the animals receiving ETCA showed the same general pattern of hydropic change, periportal fat and periportal focal necrosis as did the animals on the choline supplemented diet, and differed from the choline deficient groups only in that the latter showed centrolobular fat appears to be highly significant. Had the lipotropic effect of the ETCA been due to some non-specific factor which decreased caloric intake or metabolic rate, it would not have been expected that these animals would show the signs of protein deficiency, but would have had relatively normal livers.

The factors involved in regulating the phospholipid content of liver are ill understood. Campbell and Kosterlitz (1952) reported that the total lipid phosphorus content of rat liver is determined mainly by the level of dietary amino acids and not by the choline content of the diet. Foà, Weinstein and Kleppel (1948) and Fishman and Artom (1944a) previously stated that the total phospholipids were not affected by choline administration. However, Patterson, Keevil and McHenry (1944) did find an increased concentration of phospholipid in liver and kidney after administration of choline to deficient rats. In the present experiments the administration of ETCA produced an increased concentration of phospholipid in the liver. The increase (calculated on gm. wet liver basis) was approximately 20 p.c. in Experiment B in the choline supplemented group. A similar increase occurred in Experiment D with choline deficient animals. In Experiment C the increase was approximately 50 p.c. with choline deficient animals.

The ratio of lipid choline to total phospholipid is reduced during choline deficiency but choline administration to deficient rats can raise the ratio to normal values (see Fishman and Artom, 1944b; Foà, Weinstein and Kleppel, 1948; DiLuzio and Zilversmit, 1953). However, ETCA administered to choline deficient rats, while increasing liver phospholipid, did not increase the relative amount of choline in the phospholipid. In this respect the lipotropic action of ETCA does not appear to be due to an aggregation of extrahepatic choline

in the liver. Nevertheless, the extra phospholipids may be important in clearing the liver of the other excess lipid.

It is possible that the reduced liver lipids seen in the present experiments after ETCA administration may partly be the result of decreased lipid transport to the liver. For example, Morgan and Lewis (1953) have reported that choline deficient rats also deprived of pantothenic acid failed to develop fatty livers and concluded that due to decreased hormone production by the adrenals, lipid transport to the liver was depressed and consequently a lower liver lipid resulted.

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OVARIAN TRANSPLANTS IN OVARIECTOMISED MICE

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SUMMARY.

Survival of intrastrain ovarian homografts in ovariectomised randomly bred albino mice has been investigated. Seventy-five percent of homografts were functional for some time. The average time from grafting to the appearance of the first oestrous smear was 14 days, but this was extremely variable.

Functional activity was examined in relation to age of donor and recipient, administration of gonadotrophins, and repeated grafts in the same animal. The results emphasise the difficulty of assessing the potential survival of individual grafts.

INTRODUCTION.

It is now becoming clear that a set of propositions which may describe the results of experiments involving skin transplants to the same animal (autotransplants), to animals of the same inbred strain (isotransplants), to animals of the same randomly bred strain (intrastrain homotransplants), to animals of different strains (interstrain homotransplants), or to animals of different species (heterotransplants) is not necessarily applicable to similar experiments involving ovarian transplants (Billingham and Parkes, 1955; Krohn, 1955; Barnes and Krohn, 1957). Many reports of successful intrastrain ovarian homografts in rats and mice, for example (Harris and Eakin, 1949; Huseby and Bittner, 1951; Billingham and Parkes, 1955; Ingram and Krohn, 1956; Parkes, 1956; Deanesly, 1956) presumably indicate either that the immune reaction to such grafts is qualitatively different from that with skin or that the progress of the immune reaction may be influenced by factors peculiar to the ovary as an endocrine organ.

The obvious approach to this problem, i.e. estimation of the number and antigenicity of the histocompatibility genes which may be involved, is severely hindered by the difficulty of assessing the viability of the grafted tissue since a number of factors are known to influence the function of an ovarian transplant. These factors include the presence or absence of the hosts' ovaries (Harris and Eakin, 1949; Stevens, 1955; Deanesly, 1956), age of recipient (Deanesly, 1956),

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site of implant (Hernandez, 1943; Bielschowsky and Hall, 1953), gonadotrophins, cortisone, oestrogen (Ferguson and Kirschbaum, 1954; Krohn, 1955; Ingram and Krohn, 1956), lymphocytic infiltration (Harris and Eakin, 1949; Deanesly, 1956) and vascularization (Sturgis and Castellanos, 1957).

The present paper presents further data on some of the factors which influence survival of intrastrain ovarian homografts in ovariectomised mice.

Methods.

Animals. Randomly bred female albino mice of the SW (Sydney White) strain, both immature and adults, were used as recipients and donors.

Gonadotrophin. Pregnant mares' serum gonadotrophin (Gestyl-Organon Ltd.) was dissolved in isotonic saline and injected subcutaneously.

Technique of transplanting. Ovariectomy was carried out under light Avertin (May and Baker) anaesthesia. The ovaries were cleaned of fat and ovarian capsule in isotonic saline under a binocular dissecting microscope. The whole ovaries were transplanted ½-2 minutes after ovariectomy under the skin in the lumbar region. The underlying fascia and muscles were stroked with a pair of sharp-pointed forceps before transplanting.

Assessment of grafts. Cyclical vaginal changes as determined by daily vaginal smears were taken to indicate cyclical changes in the ovary. Where possible (Expt. II), the uterine weights were recorded and histological examination of the transplants was carried out. For histological examination the ovary was cut at 8 μ and stained with Heidenhain's haematoxylin and van Gieson's eosin. The sections were examined for primordial follicles, normal graafian follicles, atretic or cystic follicles, and normal, ageing or cystic corpora lutea. The amount of lymphocytic infiltration and invasion by connective tissue was noted in a general description of the transplant.

Experimental and Results.

Experiment I.

The following three groups of mice were selected at random from numerous mice of similar size and age:—

Group	*Number*	*Age*	*Body Weight*
A	40	9 weeks	26 gm.
B	30	6 weeks	20 gm.
C	30	3 weeks	10 gm.

Since SW mice normally reach puberty at 20 gm. group B represents pubescent mice. The treatment sub-groups each comprised ten mice (Table 1). Ten mice in group A received autografts and all other mice received homografts. The homografts were distributed so that each of three sub-groups of ten mice in any age group received ovaries from different age groups. Thus the experiment involving homografts was a 3^2 factorial in design. The mice were housed five per cage. Vaginal smears were taken daily for three weeks after the transplants, which were carried out on the same day. There was then a two-week period without smearing followed by a ten-day period of smears.

In Table 1 is listed the number of mice in each group which had commenced

TABLE 1.

Results obtained in Experiment 1.

Sub-group	No. of mice	Type of graft	Recipient	Donor	No. cycling at 3 weeks	No. cycling at 6-7 weeks
1	10	Autograft	A	A	9	9
2	10	Homograft	A	A	8	10
3	10	Homograft	A	B	7	8
4	10	Homograft	A	C	5	9
5	10	Homograft	B	A	7	9
6	10	Homograft	B	B	8	7
7	10	Homograft	B	C	9	9
8	10	Homograft	C	A	4	6
9	10	Homograft	C	B	2	8
10	10	Homograft	C	C	5	8

Analysis of variance — homografts.

Source of variation	D.F.	Mean square
3 weeks v 6 weeks (T)	1	1009***
Recipients (R)	2	662***
Donors	2	131
Interaction		
T×R	2	256*
Remainder	10	50
Error	∞	82·1

*** $P<0{\cdot}001$; * $0{\cdot}01<P<0{\cdot}05$.

cycling by the 21st day after grafting. In addition, there is listed the number of mice which gave positive smears during the final 10-day period of smearing. Three mice, one in each sub-group 1, 3 and 6, showed an oestrous cycle before three weeks, but not at 6-7 weeks. These, however, have not been excluded from the data. The data were converted to angles and submitted to analysis variance, a summary of which is appended to the Table.

It is apparent that the immature mice had fewer cycles prior to three weeks and although the differences were not large they had significantly fewer cycles at 6-7 weeks. In general, however, the age of recipient and age of donor did not greatly affect the proportion of successful grafts so that in 74 of 90 mice, ovarian homografts were apparently functional at 6-7 weeks after grafting.

In normal cycling mice an oestrous period is marked by one or two, occasionally more, fully oestrous smears, i.e. non-nucleated epithelial cells only. During the final 10-day period of smearing the length of the oestrous periods was recorded. The frequency distribution is as follows:

	Length of oestrous period (days)				
	1	2	3	4	5+
Number of periods	42	32	20	12	17

Hence, of 123 oestrous periods 29 or 23·5 p.c. were four or more days in length.

Mice which had not commenced cycling by three weeks after grafting but had done so by five weeks, were given an arbitrary time of commencement of 25 days. In the first seven groups there were a total of 11 mice in this category and they were well distributed throughout the groups. The mean latent periods for the mice in each group are shown in Table 2. In general, the latent periods

TABLE 2.

Latent periods in mice in Experiment I.

Sub-group	Age of recipient	Number of mice	Mean ± S.E. (days)
1	9 weeks	10	17·6 ± 1·44
2		10	16·8 ± 1·87
3		9	15·2 ± 2·48
4		9	16·3 ± 3·02
5	6 weeks	9	13·5 ± 2·39
6		8	13·5 ± 1·37
7		10	10·8 ± 1·68
8	3 weeks	6	21·5 ± 2·06
9		8	24·3 ± 0·55
10		8	20·9 ± 1·64

in mice 6-9 weeks of age at the time of ovarian transplants were shorter than mice three weeks old at the time of transplant. However, the variation in each group was too large to allow any further conclusion.

Experiment II.

On the day after weaning, 81 mice were ovariectomised and the ovaries autografted subcutaneously. A further 81 were ovariectomised and the ovaries from each mouse in turn were homografted subcutaneously into the next mouse. The plan of the experiment was a $2 \times 3 \times 3$ factorial, hence there were nine mice in each experimental group. The following factors were examined: (i) autografts versus homografts; (ii) three levels of pregnant mare serum gonadotrophin (PMS), 0·2, 2·0 or 20·0 I.U. injected subcutaneously in 0·2 ml. saline; (iii) gonadotrophin injected either 5, 10 or 20 days after grafting. The mice were housed in groups of five and four and were smeared for five days after the injection of gonadotrophin, not including the day following the injection.

Commencing when the mice were approximately ten weeks old, by which time they were heavier than 20 gm. and hence would be expected to have reached puberty, vaginal smears were taken for 11 days. At 4-5 months of age the mice were smeared for 14 days. By this time 76 mice originally receiving homografts and 74 with autografts were alive. The majority of the mice which died did so within a few days of the grafting operations.

Forty-two mice bearing homografts and 42 bearing autografts were killed the day following the last smear. The grafts, where present, were dissected out, fixed in buffered formalin and embedded in paraffin. The uteri were dissected

free of fat and extraneous tissue under a binocular dissecting microscope and weighed on a torsion balance after pressing on filter paper to remove lumenal fluid. At the time of examination of each mouse, its previous history was not known to the operator.

At 6-7 months of age the surviving 66 mice were divided at random into two groups each of which comprised 16 autografts and 17 homografts. The mice in one group were smeared until a prooestrous or oestrous smear was obtained when they were killed and examined as before. Males were placed with the other group, one male per female, and the females were killed seven days after a plug was observed. The mice which did not show either vaginal or psychic oestrus were killed after three weeks.

The results of the first (designated experimental) period are shown in Table 3. The analysis of the data, following conversion to angles, is appended to the Table. Since some mice died during the course of the experiment it was decided to reduce the group size to eight for the purposes of the Table and the analysis. Where there were no deaths in a group, one mouse was eliminated at random. The important feature of these results is that autografts and homo-

TABLE 3.

Results obtained in Experiment II. Mice bearing functional grafts as determined by the vaginal smear technique. There were eight mice per group (see text).

	Functional grafts:																	
	Experimental						2 months						4 months					
Levels of gonadotrophin: (i.u.)	0·2		2·0		20·0		0·2		2·0		20·0		0·2		2·0		20·0	
Time when PMS injected:	A*	H†	A	H	A	H	A	H	A	H	A	H	A	H	A	H	A	H
5 days	0	1	2	3	3	3	7	7	6	4	8	4	7	6	6	4	8	3
10 days	2	1	7	7	6	6	7	5	8	5	7	5	8	5	8	5	7	5
20 days	2	1	8	6	7	6	5	6	8	5	8	6	7	4	8	3	8	6

* Autografts; † Homograft.

Analysis of variance of the data for experimental period.

Source of variation	D.F.	Mean square
Autografts v homografts	1	48
Levels of gonadotrophin		
Linear	1	3169***
Quadratic	1	1849***
Times of injection		
Linear	1	2514***
Quadratic	1	378
Remainder	12	112
Error	∞	102·6

*** $P<0·001$.

grafts appear to respond to PMS equally well, the actual response, however, depending on the amount of PMS injected and the time of injection after grafting.

When we examine records of the same mice some months later (Table 3) it is clear that the treatment with PMS was of no importance, nor was the time of injection of the PMS. The origin of the graft, however, was important, since over two-fifths of the homografts as against approximately 7 p.c. of the autografts were non-functional when examined four months after grafting. The proportion of functional grafts at 4 and 6 months is summarised in Table 4. Only two

TABLE 4.

Summary of survival of ovarian grafts in Experiment II.

	Homografts		Autografts	
	at 4 months	at 6 months	at 4 months	at 6 months
Functional	23	19	40	29
Non-functional	19	15	2	3
Total	42	34	42	32

mice had homografts which were functional at four months, as judged by vaginal smears, but which had been destroyed at 6 months as judged by vaginal smears, and by examination of the site of grafting.

Of the 42 mice killed four months after homotransplantation, 23 possessed functional grafts which were histologically similar to the autotransplants. In the latter group only two out of 42 did not have a functional graft. The agreement between the vaginal smear results obtained prior to killing and the presence or absence of a functional graft was very satisfactory. Only one mouse gave a false negative and one a false positive. Apart from histological examination of the graft (see below) the uterine weights furnished clear-cut evidence of ovarian function. In the homografts, for example, the mean uterine weight (23 mice) was $72{\cdot}0 \pm 1{\cdot}8$ mg. compared with $10{\cdot}5 \pm 1{\cdot}0$ mg. for those in which the grafts had disappeared.

Further evidence of the functional activity of the ovarian homotransplants was shown by the results at six months as follows:—

	Uterine weights (homografts) at 6 months	
	Killed at oestrus	Killed one week after vaginal plugs
Functional	$117 \pm 15{\cdot}3$ (8 mice)	$81{\cdot}5 \pm 5{\cdot}0$ (11 mice)
Non-functional	$12{\cdot}5 \pm 0{\cdot}4$ (9 mice)	$14{\cdot}0 \pm 1{\cdot}0$ (6 mice)

The increased size of the uteri at oestrus as compared to dioestrus (pseudo-pregnancy) is strong evidence of normal follicular development and steroid production.

All grafts in this experiment were examined histologically. It was not possible, however, to draw any definite conclusions from the results. There were no obvious differences between homografts and autografts. They all had numerous corpora lutea, of varying ages, considerable early follicle development and in most cases normal antrum formation was evident. Occasionally premature luteinization and atresia were seen. Cystic formation was evident in many ovaries, some follicles reaching 5 mm. diameter.

By killing mice at oestrus it was hoped to observe recent ovulation, but this possibility was not realised. The mice were killed too soon for fresh corpora lutea formation and it was not possible to detect with any degree of certainty which follicles had ovulated or were about to ovulate. Judging by the enclosure of each graft in a fibrous tunic it seems likely that ova would have remained trapped in the corpora lutea.

Experiment III.

Forty mice received ovarian homografts one day after weaning at which time they were 20 days old. The plan of the experiment was that at intervals of approximately one month the ovary transplants in these mice be removed and replaced by ovaries from recently ovariectomised mice. This was carried out three times after the original transplants. Three weeks after the first series of transplants the mice were each given a single injection of 2 I.U. PMS and smeared for the following five days. This was done because some of the mice at this stage had not reached puberty. After each of the next three transplants the mice were smeared for 8-10 days prior to replacement with a fresh graft. Hence, except in the case of the initial graft the functional activity of the grafted ovary was assessed by cyclical vaginal changes prior to 28-30 days. From the results of Exp. I, it might be expected that a small proportion of grafts may not have begun cycling by this time. This was found to be so in five cases, but since the ovary appeared normal these were classed as functional.

During the course of the experiment three mice gave positive smears but no ovary was present when the mice were examined 3-5 days later. It is possible that a rapid homograft reaction had taken place between the time of the positive

TABLE 5.

Results obtained in Experiment III.

	Period ending	Functional grafts*	Non-functional grafts
I	4.3.58	27	6
II	3.4.58	25	8
III	2.5.58	26	7
IV	2.6.58	23	10

* Based on vaginal smears except in five cases based on examination of the graft.

$\chi^2_{(3)} = 1 \cdot 12 \quad 0 \cdot 7 < P < 0 \cdot 8.$

smear and the time of examination or these may have been false positives. Nevertheless, they were classed as negative.

The results are summarised in Table 5. Seven mice died during the course of the experiment and their records were discarded. It is apparent that there were no significant differences in the proportion of functional grafts at the different periods, 76·5 p.c. of mice bearing functional ovarian homografts at 28-30 days.

It was important to determine whether the same mice continually rejected grafts. The data were therefore examined for heterogeneity (Table 6). The

TABLE 6.

Results obtained in Experiment III (cont.)

Number of non-functional grafts	Coefficients	Expected		Observed	
0	0000	11·309		14	14
1	0001	3·472		2	
	0010	3·472		3	
	0100	3·472	13·89	3	10
	1000	3·472		2	
2	0011	1·066		0	
	0101	1·066		0	
	1001	1·066		1	
	0110	1·066	6·4	4	6
	1010	1·066		0	
	1100	1·066		1	
3	0111	0·327		0	
	1011	0·327		0	
	1101	0·327	1·3	3	3
	1110	0·327		0	
4	1111	0·100		0	0

$\chi^2_{(4)} = 3·99$ $\quad 0·3 < P < 0·5.$

sixteen possible combinations of accepted (0) or rejected (1) grafts are set out in Table 6 together with the proportion of mice which would be expected to fall into each class on the assumption that 31 out of 132 grafts were rejected at random. The observed results are clearly not significantly different from expectation and hence there is no reason to discard the hypothesis that the grafts were rejected at random.

DISCUSSION.

Approximately three in every four heterotopic (subcutaneous) ovarian homotransplants in ovariectomised mice which have reached puberty in the randomly-bred colony in this Department may be expected to bring about cyclical vaginal changes. The average time from grafting to the appearance of the first oestrous vaginal smear is 14 days. The time, however, is extremely

variable, in some cases being longer than four weeks. In immature mice the appropriate dose of gonadotrophin given 10 days after grafting brings about an oestrous response in all mice which are apparently likely to accept the graft, whether for a longer or shorter time. The wide variability in latent periods in adult mice may thus be due to differences in the level of endogenous gonadotrophin which reach the transplant. Whether this in turn could be due to differences in pituitary output or to differences in rate or completeness of vascularisation of the graft is not known.

Parkes (1956) raised the question of the relationship of the latent period to survival of grafts. Apart from the fact that grafts which never function have an infinitely long latent period, there is no reason to refute the suggestion that the length of the latent period is unrelated to survival. In our experiments immature recipients seem to have as high a proportion of takes as mice which have recorded an oestrous smear following early gonadotrophin administration.

The results following gonadotrophin administration raise an important question. Whilst exogenous gonadotrophin can stimulate oestrogen production, in some cases as early as five days after the graft, there is no evidence that this stimulation affects the subsequent history of the graft in any way. Therefore, we may well ponder what is the period of time after grafting which must elapse for ovarian function to be compatible with survival. The homograft reaction may clearly overwhelm the transplant at any time, although the majority of grafts which are functional at 4-6 weeks may be expected to survive indefinitely. Hence, while the future survival of a population of ovarian homografts may be determined by cyclical function at 4-6 weeks, the response of an individual graft cannot be accurately assessed at any time.

The question of whether or not ovarian tissue is qualitatively different from skin either in eliciting or resisting the immune reaction must be settled before the assessment of individual grafts can be further investigated. The results of Experiment III at least suggest that the degree of incompatibility between our mice is slight and hence fairly rapid progress may be expected in estimating the number and importance of the histocompatibility genes involved.

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THE USE OF SPECIFIC ANTITOXIN AGAINST EXPERIMENTAL STAPHYLOCOCCAL INFECTION IN MICE

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SUMMARY.

Attempts of most workers to protect mice with specific antitoxin against experimental staphylococcal infection have failed.

Mouse immunisation experiments described above show that staphylococcal antiserum will protect mice against death from experimental infection if—

(1) the challenge, and

(2) the interval between administration of serum and challenge

are appropriately chosen. Mice are "sensitized" if the interval is small.

Neglect of one or both of these considerations would account for the discrepancies in the literature.

INTRODUCTION.

Reports on clinical trials of staphylococcal alpha antitoxin are conflicting. Whilst Baker and Shands (1939) obtained excellent results in osteomyelitis and staphylococcaemia, Robertson (1938) found no benefit at all in patients treated with antitoxin. Other observers (Panton, Valentine and Dix, 1931; Dolman, 1934; Joyner and Smith, 1936; Williams, 1937; Kleiger and Blair, 1943) have reported varying degrees of success or failure.

Results of animal experiments are also contradictory. Frappier and Sonea (1956) could find only two records of successful passive immunisation of laboratory animals against challenge with lethal doses of living staphylococci. In both instances (Parish, O'Meara and Clark, 1934; Kitching and Farrell, 1936) mice were used, and they were challenged by the intraperitoneal route. Frappier and Sonea state that they could not confirm these results, but give no particulars of their experiments.

Boake (1956), also using the intraperitoneal route for challenging mice, obtained protection if antitoxin were given three hours before challenge, but none if the serum and challenge suspension in "clotting mixture" were administered together.

Using Boake's (1956) "clotting mixture" method for enhancing the virulence of the challenge culture, we obtained protection regularly if antitoxin were administered 24 hours before challenging. If, however, antitoxin were given two to seven hours before infecting the mice, protection was weak or absent.

The results of these and subsequent experiments together with some deductions drawn from them form the basis of this communication.

Materials and Methods.

Experimental animals. White mice weighing 18-22 gm. were used.

Sera used.

(a) Staphylococcal alpha antitoxin, Batch 65/66 (730 units/ml.) prepared in horses.
(b) Diphtheria antitoxin, concentrated and refined, in the same manner as (a).
(c) Normal horse serum.
(d) Human gamma globulin as prepared at the Commonwealth Serum Laboratories.

All dilutions of serum or gamma globulin were made in normal saline.

Administration of sera. In early experiments serum or gamma globulin was administered intravenously; later, it was given subcutaneously. The volume injected was always 0·5 ml.

Antihistamines and corticotrophin (A.C.T.H.).

"Antistine" (Ciba) two doses each of 0·1 ml. of 1:5 dilution in saline were given subcutaneously.

Corticotrophin (A.C.T.H.) in gelatin solution was injected subcutaneously in doses varying from 1 unit to 5 units.

Active immunisation of mice. Mice were immunised as previously described (North, 1958) with two subcutaneous injections, given one week apart, of diluted staphylococcal toxin, and challenged two weeks after the second dose.

Challenge strain of staphylococcus. A coagulase-producing strain of *Staphylococcus pyogenes* (S 1531) isolated from a fatal case of staphylococcal meningitis was used throughout. Stock cultures were maintained on veal infusion agar under paraffin.

Preparation of suspension of staphylococci for challenge.

Four different methods of preparing the challenge suspension were used.

(a) In modification of Boake's clotting mixture (1 p.c. human fibrinogen and 1 p.c. fresh human plasma. North, 1958).
(b) In 5 p.c. hog's gastric mucin.
(c) In normal saline.
(d) An overnight culture in veal infusion broth.

Suspension in (a) and in (b) are methods described for enhancing the virulence of staphylococci (Boake, 1956; Nungester, Wolf and Jourdonais, 1932). Either an overnight culture of S 1531 on veal infusion agar or material from a stable living bacterial pool (North, 1958) was used for preparing suspensions in Boake's "clotting mixture" (a). Both gave substantially the same results.

An overnight culture on veal infusion agar was used for preparing suspensions in hog's gastric mucin (b) in normal saline (c) and for inoculating veal infusion broth (d) (1 loopful into 100 ml. of broth in a 200 ml. conical flask).

Mice were challenged by the intraperitoneal route (1 ml. of suspension (a), (b) or (c), or 0·6 ml.-1·0 ml. of broth culture (d)).

The number of living staphylococci in the various challenge suspensions used (as determined prior to each challenge by the method of Miles and Misra, 1938) were of the following order:

(a) In Boake's clotting mixture .... 150×10^6/ml.
(b) In 5 p.c. hog's gastric mucin .... 12×10^6/ml.
(c) In saline .... 4000×10^6/ml.
(d) Broth culture .... $500\text{-}750 \times 10^6$/ml.

Challenge suspensions of the above strengths and volumes usually killed 80 p.c. or more of control mice within 24 hours. This represented about 4 LD_{50} as judged by giving graduated challenge doses to groups each of 4 normal mice.

Antitoxin content of mouse and horse sera. Mice were exsanguinated under ether-chloroform anaesthesia and antitoxin was estimated by the method of Burnet (1931) against International Standard antitoxin. The antitoxic content of horse serum was also estimated by Burnet's (1931) method.

Haemolysin. Haemolysin content of supernatant of overnight broth culture was estimated by Burnet's (1931) method after spinning down the bacteria and subsequent Seitz filtration.

RESULTS.

Experiments using challenge suspension in Boake's "clotting mixture".

Antitoxin administered on the day before challenge by Boake's method regularly protected mice and a well-graded response was obtained. The results of a representative experiment are shown in Table 1. The antitoxin was injected intravenously. It will be noticed that as little as 13 units of antitoxin afforded some protection.

TABLE 1.

Protective action of staphylococcal antitoxin given 24 hours before challenge with staphylococci suspended in Boake's "clotting mixture".

Dose of antitoxin	Date of death	Survivors after 7 days
Nil (controls)	1, 1, 1, 1, 1, 1, 1, 1, 1	1/10
13 units.	1, 1, 1, 1, 1, 1, 2, 2, 2	2/10
40 units	1, 1, 1, 1, 2, 2	4/10
122 units	2, 2, 4	7/10
365 units	2, 5	8/10

Antitoxin was administered intravenously. Mice were challenged by intraperitoneal route.

TABLE 2.

Antitoxin levels in mouse serum at different time intervals after injection of 40 units of staphylococcal alpha antitoxin (A.T.) by different routes.

Injection route of antitoxin	Time of bleeding mice after injection	Antitoxin level of mouse serum
Intravenous (I.V.)	2 hours	15 units/ml.
Intravenous (I.V.)	6½ hours	12 units/ml.
Intravenous (I.V.)	24 hours	5 units/ml.
Subcutaneous (S. cut)	2 hours	1 unit/ml.
Subcutaneous (S. cut)	6½ hours	3 units/ml.
Subcutaneous (S. cut)	24 hours	5 units/ml.

15 mice were injected I.V. and 15 mice S. cut. with 40 units A.T. Then 5 of each group were bled out 2, 6½ and 24 hours later. Equal volumes of serum from each of the 5 mice of a subgroup were pooled and the A.T. level determined.

There is a considerable fall in the serum antitoxin level 24 hours after staphylococcal antitoxin is administered intravenously to mice (Table 2). However, when mice were challenged by Boake's method two to three hours after the administration of antitoxin, little protection could be demonstrated. The results of a typical experiment are shown in Table 3. The morning following

TABLE 3.

Effect of staphylococcal antitoxin given 2½ hours before challenge with staphylococci suspended in Boake's "clotting mixture".

Dose of antitoxin	Date of death	Survivors after 7 days
Nil (Controls)	1, 1, 1, 1, 1, 1. 1, 2, 2	1/10
4 units	1, 1, 2, 2, 2, 2, 2, 2, 2	1/10
13 units	1, 1, 2, 2, 2, 2, 2, 6	2/10
40 units	1, 1, 1, 2, 2, 2, 2, 2	2/10
122 units	1, 1, 1, 2, 2, 2, 5	3/10

Antitoxin was administered intravenously. Mice were challenged by intraperitoneal route.

challenge, the surviving mice of those groups receiving small doses of antitoxin looked less sick than those receiving larger doses. The general pattern was always the same – the larger the dose of serum the mice received shortly before challenge the poorer were the results.

Table 4 shows the effect of the route and time of administration of a large dose of antitoxin (0·5 ml. of the undiluted concentrated serum) in relation to

TABLE 4.

Effect of route and time of administration of a large dose (365 units) of antitoxin (A.T.) in relation to challenge with staphylococci suspended in Boake's "clotting mixture".

Route of injecting A.T.	Time of administration of A.T.	Day of death	Survivors after 7 days
Nil	(Neg. controls)	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0/12
Intravenous	24 hrs. before challenge	1, 2, 2, 7	8/12
Intravenous	2 hrs. before challenge	1, 1, 1, 1, 1, 1, 1, 1, 2, 2, 5	1/12
Intravenous	2½ hrs. after challenge	1, 1, 1, 1, 1, 1, 1, 2, 6	3/12
Subcutaneous	24 hrs. before challenge	5	11/12
Subcutaneous	2 hrs. before challenge	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0/12
Subcutaneous	2½ hrs. after challenge	1, 1, 1, 1, 1, 1, 1, 6, 7	3/12

Mice were challenged by intraperitoneal route.

the challenge by Boake's method. In spite of the difference in serum antitoxin levels after intravenous and subcutaneous injection of identical amounts of staphylococcal antitoxin (Table 2), it is apparent that using either route of administration, protection is good if serum is given 24 hours before, poor or absent if given two hours before challenge. When antitoxin is administered 2½ hours after challenge, the mice actually fare better than when it is given two hours before the infecting dose.

The possibility that some phenomenon, allergic in nature, played a part in the failure of antitoxin to protect when given up to seven hours before challenge was explored. Two doses of "antistine", the first injected a few minutes after administration of antitoxin and the second one hour after intraperitoneal challenge, made no difference to the results.

The effect of corticotrophin (A.C.T.H.) was also tried. The results of one of the two experiments carried out are shown in Table 5. The results of

TABLE 5.

Effect of corticotrophin (A.C.T.H.) on staphylococcal antitoxin (A.T.) given 2 hours before challenge with staphylococci suspended in Boake's "clotting mixture".

Treatment	Day of death	Survivors 7 days after challenge
Nil (Negative controls)	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0/12
Staph. A.T. 24 hrs. before challenge	2, 3, 4, 4, 5	7/12
Staph. A.T. 7 hrs. before challenge	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0/12
Staph. A.T. 2 hrs. before challenge	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 2	0/12
Staph. A.T. 2 hrs. before challenge, also 5 units A.C.T.H.	1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 3, 4	0/12
Staph. A.T. 2 hrs. before challenge, also 1 unit A.C.T.H.	1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 3, 3	0/12

All injections of staph. A.T. (365 units) and of A.C.T.H. were made subcutaneously. A.C.T.H. was injected at a separate site 1 hour before intraperitoneal challenge.

the other test were even less convincing. A.C.T.H. may modify slightly the adverse effect of injecting antitoxin shortly before challenge by Boake's virulence-enhancement method. However, the degree of protection produced is small compared with that shown by the mice passively immunised 24 hours before challenge.

To test the possibility that a large dose of antitoxic serum given a few hours before challenge decreased the resistance of the animal to infection rather than that it simply failed to protect, mice actively immunised with two injections of toxin were used (Fisher, 1957; North, 1958). Two weeks after the second

TABLE 6.

Effect of administering a large dose of staphylococcal antitoxin (A.T.) to actively immunised mice 2 hours before challenge with staphylococci suspended in Boake's "clotting mixture".

Status of mice used	Treatment 2 hours before challenge	Survivors 24 hours after challenge
Actively immunised	Nil	13/14
Actively immunised	365 units staph. A.T.	9/14
Normal controls	Nil	8/18
Normal controls	Normal horse serum	6/18

Staphylococcal antitoxin (A.T.) and normal horse serum (both undiluted 0·5 ml.) were given subcutaneously. Mice were challenged by the intraperitoneal route.

injection of toxin they were divided at random into two groups – one group was given antitoxin, the other group was given none. Two hours later both groups together with suitable control mice were challenged by Boake's method. As Table 6 shows, the actively immunised mice treated with antitoxin were only poorly protected whilst the untreated actively immunised mice were protected to a statistically significant degree compared with the non-immunised controls. ($P < 0{\cdot}05$.) For reasons discussed below, the results of this and subsequent experiments were read 24 hours after challenge.

Experiments along similar lines, but using diphtheria antitoxic serum instead of staphylococcal antitoxin, were also carried out. Diphtheria antitoxin given 24 hours before challenge with living staphylococci by Boake's method was found to protect, sometimes to a significant degree. When it was administered two hours before challenge, it failed to protect normal animals and depressed the resistance of mice actively immunised with staphylococcal toxin.

Experiments using challenge suspension in 5 p.c. hog's gastric mucin.

Preliminary experiments showed that as with Boake's method, protection was easy to demonstrate when antitoxin was given 24 hours before challenge with staphylococci suspended in 5 p.c. hog's gastric mucin, but not when it was injected two hours before challenge (Table 7). The results of a representative experiment in which graded doses of antitoxin were given 24 or two hours before challenge are shown in Table 8. The pattern of protection is similar to that seen when Boake's challenge method was used (see Tables 1 and 3). There was a well-graded response to antitoxin injected 24 hours before challenge. On the other hand, mice given antitoxin two hours before challenge fared progressively worse as the dose was increased. There is a statistically significant difference in favour of the group getting 13 units of antitoxin two hours before challenge, as compared with those getting the same dose 24 hours before being infected. ($P < 0{\cdot}05$.) With a dose of 365 units of antitoxin (undiluted serum) the position is completely reversed, namely, a statistically highly significant difference in favour of the "24 hours" group. ($P < 0{\cdot}005$.)

TABLE 7.

Effect of time of administration of large dose of staphylococcal antitoxin (A.T.) in relation to challenge with staphylococci suspended in 5% hog's gastric mucin.

Treatment	When treatment given	Survivors 24 hrs. after challenge
Nil	(Neg. controls)	0/12
Normal horse serum	24 hrs. before challenge	2/12
Normal horse serum	2 hrs. before challenge	2/12
365 units staph. A.T.	24 hrs. before challenge	10/12
365 units staph. A.T.	2 hrs. before challenge	0/12

Normal horse serum and staph. A.T. injected subcutaneously. Mice challenged by intraperitoneal route.

TABLE 8.

Effect of time of administration of graded doses of staphylococcal antitoxin (A.T.) in relation to challenge with staphylococci suspended in 5% Hog's gastric mucin.

Treatment	When treatment given	Survivors 24 hrs. after challenge
Nil	(Neg. controls)	3/12
Normal horse serum	24 hrs. before challenge	3/12
13 units staph. A.T.	24 hrs. before challenge	2/12
40 units staph. A.T.	24 hrs. before challenge	5/12
122 units staph. A.T.	24 hrs. before challenge	8/12
365 units staph. A.T.	24 hrs. before challenge	10/12
Normal horse serum	2 hrs. before challenge	5/12
13 units staph. A.T.	2 hrs. before challenge	7/12
40 units staph. A.T.	2 hrs. before challenge	3/12
122 units staph. A.T.	2 hrs. before challenge	3/12
365 units staph. A.T.	2 hrs. before challenge	1/12

Normal horse serum and staph. A.T. injected subcutaneously. Mice challenged by intraperitoneal route.

Experiments using challenge suspension in normal saline.

When challenging with a suspension of staphylococci in normal saline, it was found relatively easy to demonstrate protection in mice given antitoxin either two hours or 24 hours beforehand. However, as the results of the experiment shown in Table 9 suggest, the mice are adversely affected to some extent by large doses of serum given shortly before challenge. This effect is shown better in an experiment in which graded doses of serum were used (Table 10). With doses of antitoxin up to 40 units, protection was better when it was given two hours before challenge than when given 24 hours before. When the dose was 122 units (Table 10) or 360 units (Table 9), the results were reversed, the "24 hours" mice being better protected.

TABLE 9.

Effect of time of administration of large dose of staphylococcal antitoxin (A.T.) in relation to challenge with staphylococci suspended in normal saline.

Treatment	When treatment given	Survivors 24 hrs. after challenge
Nil	(Neg. controls)	1/12
365 units staph. A.T.	24 hrs. before challenge	9/12
40 units staph. A.T.	2 hrs. before challenge	8/12
365 units staph. A.T.	2 hrs. before challenge	5/12

Staph. A.T. injected subcutaneously.

Mice challenged by intraperitoneal route.

TABLE 10.

Effect of time of administration of graded doses of staphylococcal antitoxin in relation to challenge with staphylococci suspended in normal saline.

Treatment	When treatment given	Survivors 24 hrs. after challenge
Nil	(Neg. controls)	2/12
Diph. A.T. (undiluted)	2 hrs. before challenge	2/12
13 units staph. A.T.	24 hrs. before challenge	2/12
40 units staph. A.T.	24 hrs. before challenge	6/12
122 units staph. A.T.	24 hrs. before challenge	11/12
13 units staph. A.T.	2 hrs. before challenge	5/12
40 units staph. A.T.	2 hrs. before challenge	10/12
122 units staph. A.T.	2 hrs. before challenge	7/12

Diph. A.T. and staph. A.T. injected subcutaneously.

Mice challenged by intraperitoneal route.

Experiments using an overnight broth culture for challenge.

More difficulty was experienced with an overnight broth culture in selecting a suitable challenge dose, namely, one that represented about $4 \times LD_{50}$, than was the case with challenge suspensions prepared from a culture on veal infusion agar. However, the results of an experiment (Table 11) where the challenge of 1 ml. was considerably greater than $4 \times LD_{50}$ show that protection can be demonstrated in mice given a large dose of antitoxin (365 units) either 24 hours or two hours before challenge. Groups of mice given gamma globulin, diluted 1:5, were also included in this experiment. Neither in this nor in other tests has gamma globulin, irrespective of dosage, given significant protection against challenge with living staphylococci.

TABLE 11.

Effect of time of administration of large dose of staphylococcal antitoxin (A.T.) or of gamma globulin in relation to challenge with an overnight broth culture of staphylococci.

Treatment	When treatment given	Survivors 24 hrs. after challenge
Nil	(Neg. controls)	0/12
Normal horse serum	24 hrs. before challenge	2/12
Normal horse serum	2 hrs. before challenge	1/12
1:5 gamma globulin	24 hrs. before challenge	1/12
1:5 gamma globulin	2 hrs. before challenge	1/12
365 units staph. A.T.	24 hrs. before challenge	9/12
365 units staph. A.T.	2 hrs. before challenge	7/12

Normal horse serum, gamma globulin and staph. A.T. injected subcutaneously.

Mice challenged with 1 ml. of broth culture by intraperitoneal route.

The protocol of an experiment in which graded doses of antitoxin over a wide range were given either 24 or 2 hours before challenge is shown in Table 12. In this case the challenge was somewhat less than $4 \times LD_{50}$ and a better

TABLE 12.

Effect of time of administration of graded doses of staphylococcal antitoxin (A.T.) in relation to challenge with an overnight broth culture of staphylococci.

Treatment	When treatment given	Survivors 24 hrs. after challenge
Nil	(Neg. controls)	1/12
Normal horse serum	24 hrs. before challenge	2/12
13 units staph. A.T.	24 hrs. before challenge	6/12
40 units staph. A.T.	24 hrs. before challenge	11/12
122 units staph. A.T.	24 hrs. before challenge	11/12
365 units staph. A.T.	24 hrs. before challenge	11/12
Normal horse serum	2 hrs. before challenge	1/12
13 units staph. A.T.	2 hrs. before challenge	5/12
40 units staph. A.T.	2 hrs. before challenge	8/12
122 units staph. A.T.	2 hrs. before challenge	10/12
365 units staph. A.T.	2 hrs. before challenge	11/12

Normal horse serum and staph. A.T. injected subcutaneously.

Mice challenged with 0·75 ml. of broth culture by intraperitoneal route,

graded response was shown by the "2 hours" mice than by those dosed the day before challenge.

No toxin was demonstrated in the supernatant of the broth culture used as challenge in this experiment, nor in the supernatant of cultures used in other similar trials. Further, mice injected intravenously with 1 ml. of Seitz filtered supernatant showed no ill effects whatever.

TABLE 13.

Effect of administering a large dose of staphylococcal antitoxin (A.T.) to actively immunised mice 24 or 2 hours before challenge with an overnight broth culture of staphylococci.

Status of mice used	Treatment before challenge	Survivors 24 hrs. after challenge
Actively immunised	365 units staph. A.T. 24 hours before	12/13
Actively immunised	365 units staph. A.T. 2 hours before	13/14
Actively immunised	Nil	14/14
Normal (controls)	365 units staph. A.T. 24 hours before	14/15
Normal (controls)	365 units staph. A.T. 2 hours before	14/16
Normal (controls)	Nil	4/16

Staph. A.T. (undiluted 0·5 ml.) injected subcutaneously.

Mice challenged with 0·9 ml. of broth culture by intraperitoneal route.

The results of an experiment in which mice previously actively immunised together with unimmunised mice were given antitoxin before challenge with an overnight broth culture of staphylococci are shown in Table 13. Unlike the actively immunised mice challenged by Boake's virulence-enhancement method (see Table 6), administration of 365 units of antitoxin 2 hours before injection had little or no adverse effect on the survival rate. The actively immunised mice survived whether they had antitoxin 2 or 24 hours before challenge, or no antitoxin at all. Mice that were not actively immunised beforehand were, like others challenged with broth culture (Tables 11 and 12), protected to a statistically significant degree by antitoxin given either 2 or 24 hours beforehand.

DISCUSSION.

The results presented above show clearly the importance of the time interval between administration of staphylococcal antitoxic serum and the challenge suspension in mouse protection tests. Using either Boake's "clotting mixture" or hog's gastric mucin to enhance the virulence of the infecting staphylococci, the protective effect of antitoxin is definitely better when the serum is given 24 hours before challenge than when it is administered 2 hours before infecting the mice.

It is known that following experimental staphylococcal infection in laboratory animals (Burnet, 1929; Kleiger, Blair and Hallman, 1942), toxin is produced *in vivo*. It would, therefore, seem that the antitoxin content of the tissues some time after challenge when the invading staphylococci are elaborating toxin may be of more importance than the antitoxin level at the actual time of challenge. In this respect it should be noted (Table 4) that antitoxin given either subcutaneously or intravenously 2½ hours following challenge can still have a definite protective effect ($P < 0{\cdot}05$ in both cases versus untreated controls 24 hours after challenge), whilst the same dose of antitoxin given 2 hours before challenge by Boake's method may have none.

Further, the results of experiments in which actively immunised mice were given antitoxin 2 hours before challenge by Boake's method (Table 6) indicate that a large dose of heterologous serum actually lowers, temporarily, the resistance of the mice to the challenge.

On the other hand, further experiments show that when either a saline suspension of staphylococci or an overnight broth culture is used for challenge (Tables 9, 10, 11, 12 and 13) there is no definite evidence of the horse serum temporarily lowering the resistance of the mouse.

The nature of this "post serum" period of lowered resistance to challenge by virulence-enhancement methods is being investigated. Whatever its nature, its occurrence almost certainly accounts for the failure of some workers to protect mice passively against intraperitoneal challenge with living staphylococci (Frappier and Sonea, 1956; Boake, 1956).

It is of interest to note that neither Parish and his fellow workers (1934), nor Kitching and Farrell (1936), who both achieved some success, used measures to enhance the virulence of their challenge cultures. Parish and his team challenged 7 hours after giving antitoxin, Kitching and Farrell 20 hours after.

Besides the time interval between administration of serum and challenge, the actual dose of the latter is important in mouse protection tests. The most suitable dose appeared to be about $4 \times LD_{50}$. If, for some unknown reason, the challenge dose proved to be more highly lethal, protection could often not be demonstrated.

In our earlier experiments, Boake's method of challenge was used exclusively and the results of the protection tests read 7 days after infecting the mice. This was satisfactory, because animals that lived for twenty-four to forty-eight hours usually survived indefinitely. However, when mice were challenged with the much larger numbers of living organisms necessary in non-virulence enhancement methods (saline suspensions or broth cultures) death occurred up to a week or more later from suppurative lesions in the kidneys, although antitoxin protected them against early death. Readings were, therefore, made 24 hours after challenge as a routine, a practice followed by other workers (Kitching and Farrell, 1936; Frappier and Sonea, 1956). Elek (1959) is highly critical regarding the experimental work on the protection afforded by alpha antitoxin whether produced by active or passive immunisation. He maintains that the development of local metastatic lesions is not prevented by antitoxin and that, in general, immunisation only results in slightly longer survival of the mouse or rabbit.

We believe, however, that under certain conditions true protection may be afforded by antitoxic serum. The survivors of many experiments, such as those shown in Tables 1 and 4, have been observed for some weeks at least. Most of them have remained in good health. Similarly, mice actively immunised with toxin or toxoid and subsequently challenged by Boake's method (North, 1958) have remained well for indefinite periods.

However, it seems certain that the protection is not wholly due to alpha antitoxin *per se*. As recorded above, it has been found that diphtheria antitoxic serum in which no staphylococcal alpha antitoxin could be detected had some protective action against experimental infection with staphylococci.

A similar observation was made many years ago (Key, 1926), and the matter is now being investigated further.

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MURRAY VALLEY ENCEPHALITIS VIRUS: PREPARATION OF AN INFECTIVE "RIBONUCLEIC ACID" FRACTION[1]

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SUMMARY.

Crude suspensions of Murray Valley encephalitis virus, when treated with concentrated phenol, yielded an infective product; this has been designated "RNA".

Both virus and "RNA" were titrated on the chorioallantoic membrane of 12-day chick embryos. The embryos died in three days.

In appropriate diluents both virus and "RNA" lost 99 p.c. of their infectivity after 30 minutes at 48° C.

"RNA" was inactivated by ribonuclease, normal rabbit serum, and extracts of spleen and lung. Extracts of mouse brain and liver protected the "RNA" against inactivation of each of these agents.

INTRODUCTION.

Gierer and Schramm (1956) showed that treatment of purified preparations of tobacco mosaic virus with concentrated phenol solution at low temperatures yielded an infective agent which differed from the original virus. Thus, the infectivity of the product was destroyed by ribonuclease (RNAse), it was unstable at 37° C. and sedimented very slowly in the ultracentrifuge. These and other properties suggested that the infective product was essentially ribonucleic acid (RNA). Virus produced following infection with RNA had properties similar to those of the virus from which the RNA was derived. Phenol treatment of crude preparations of several small animal viruses have been shown to yield infective agents which have some of the properties of RNA (Colter, Bird and Brown, 1957; Colter, Bird, Moyer and Brown, 1957; Wecker and Schäfer, 1957; Cheng, 1958; Brown, Sellers and Stewart, 1958).

This paper presents evidence that an infective substance can be prepared from a crude suspension of Murray Valley encephalitis (MVE) virus by treatment with phenol. The evidence is consistent with the concept that the sub-

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stance is, or contains, RNA. In conformity with the practice adopted by other authors, we have designated the substance "RNA" in this paper, the inverted commas being used to imply that the infective material is not necessarily pure RNA. The present investigation showed that "RNA" of MVE virus resembled the "RNA" of other animal viruses in several respects. New aspects of the problem have also been studied.

Materials and Methods.

Saline. An aqueous solution of NaCl (0·15 M).

Phosphate citrate saline. An aqueous solution containing Na_2HPO_4 (0·017 M), citric acid (0·016 M) and NaCl (0·13 M) pH 7·1.

Phosphate saline. An aqueous solution containing NaH_2PO_4 (0·05 M), NaOH (0·044 M) and NaCl (0·13 M) pH 7·7.

Phenol. Phenol (A.R.) was distilled *in vacuo* and the distillate saturated with glass-distilled water. The preparation could be kept at 0° C. for several months.

Ethyl ether. Ethyl ether was shaken with a saturated aqueous solution of ferrous sulphate containing a few drops of *N* H_2SO_4. The ether layer was distilled and the distillate kept at 0° C. until required. A fresh batch was prepared each day.

Normal rabbit serum (NRS). Young adult rabbits were first tested to make sure they contained no neutralizing antibody to Murray Valley encephalitis virus. Blood was obtained from the ear vein, held at 37° C. for several hours, then chilled, and the serum separated. The serum was stored in separate lots at — 20° C. until required. The stored serum was used unheated.

Ribonuclease (RNAse). Crystalline pancreatic ribonuclease obtained from the Worthington laboratories and stated to be free of protease.

Eggs. Fertile eggs were laid by white leghorn hens run with black orpington roosters. The eggs were incubated at 39° C. until used. Just before use, they were candled and an area of the shell over the chorioallantoic membrane marked for drilling in such a way as to avoid large vessels.

Source of virus. Virus was routinely produced from the brains of suckling mice. Mice four days old were inoculated intraperitoneally with 0·05 ml. containing 10^7 baby mouse LD_{50} of Murray Valley encephalitis virus, derived from a strain isolated in 1951 (French, 1952). Four days later most of the mice were moribund. The moribund mice were killed and their brains removed either into chilled phosphate citrate saline or into a dry tube held at — 70° C. It was also possible to extract virus from chorioallantoic membranes, but the yield was smaller than from mouse brains.

Preparation of virus extract. Infected baby mouse brains were ground in the mortar described below together with sufficient phosphate citrate saline to yield a 20 p.c. (wet weight/volume) suspension which was then centrifuged at 2,000 *g* for 10 minutes. The resulting supernatant was either used fresh or stored at — 70° C.

Preparation of infective "RNA". The standard procedure for "RNA" production by phenol treatment was as follows. All steps were carried out between 0° C. and 4° C. The prefrozen (— 70° C.) infected brains were ground into phosphate citrate saline for two minutes, using a glass mortar fitted with a teflon pestle which rotated at about 4,000 r.p.m. Sufficient brains were used to give a 20 p.c. (w/v) suspension. This was centrifuged at 2,000 *g* for 10 minutes, and the supernatant treated in the mortar with an equal volume of 90 p.c. (v/v) phenol for six minutes. After centrifugation at 2,000 *g* for eight minutes, the clear supernatant was treated twice more with half the original volume of 90 p.c. phenol (mixing for two minutes, centrifugation for three minutes). The final supernatant

was extracted three times with 5 to 10 volumes of ether using the apparatus described by Keck (1956). Nitrogen was bubbled through the aqueous phase for about three minutes to remove most of the ether.

As occasion required, the standard method was varied in two ways, but titres of "RNA" were essentially the same as with the standard method. These variations were: (1) Freshly harvested infected brains were ground directly into a mixture of phosphate citrate saline and 90 p.c. phenol, and then treated as in the standard procedure. (2) Infected brains, stored at — 70° C. for varying periods, were ground directly into a mixture of equal volumes of buffered saline and 90 p.c. phenol, and then treated as in the standard procedure.

Infective "RNA" could also be prepared by treatment of chorioallantoic membranes (CAM) with phenol as above.

Method of storage. Virus and "RNA" were stored as required in sealed glass ampoules at — 70° C.

Preparation of normal RNA. "RNA" was prepared from normal suckling mouse brain in the same way as "RNA" was made from infected brains. It was used without further dilution.

Preparation of tissue extracts. Adult mice were killed by chloroform, and the organs were removed and washed in chilled saline. Organ emulsions were made in the mortar described above, centrifuged at 2,000 *g* for five minutes and either used fresh or stored at — 70° C.

RESULTS.

Titration of virus and "RNA".

The standard method of titration was to inoculate 0·05 ml. volumes of falling 10-fold dilutions onto the chorioallantoic membrane of 12-day chick embryos, six embryos being used for each dilution. The 50 p.c. lethal endpoint was determined by opening each egg at 72 hours, and this endpoint is expressed here as the negative logarithm to the base 10 of the dilution of virus or "RNA".

Ten p.c. normal rabbit serum in saline was used as diluent for virus, and phosphate citrate saline (pH 7·1) or phosphate saline (pH 7·7) as diluent for "RNA". Under these conditions virus preparations titred at about 6·5 logs, and "RNA" made by the standard technique titred at about 4·5 logs; the endpoints were generally sharp. Variations from the standard titration technique produced different endpoints for both virus and "RNA". These variations were as follows:

Routes of inoculation into eggs and mice. Chick embryos of 12 days' incubation were inoculated onto the chorioallantoic membrane or into the amniotic or suballantoic cavities. Six-day-old embryos were used for yolk sac inoculation. Suckling mice were between three and six days of age when inoculated. In each case the inoculum was 0·05 ml., except for intracerebral inoculation of mice for which 0·01 ml. was used. Allowance was made for this smaller inoculum when preparing the results of Table 1, which are calculated on the basis of an inoculum of 0·05 ml. Deaths of chick embryos were determined by opening the eggs at a fixed time, which was three days in the case of eggs inoculated on the chorioallantois. Baby mice were observed for 14 days: the great majority of deaths after dilute inocula occurred on the 6th and 7th days.

TABLE 1.

Titres of standard preparations of virus and "RNA", estimated by various procedures.

Host	Route of inoculation	Virus titre	RNA titre	V/R* ratio	Observed (days)
Chick Embryo	Chorioallantois	6·5±	4·8	1·7	3
	Yolk sac	6·5	2·5	4·0	5
	Allantoic cavity	4·8	1·8	3·0	6
	Amniotic cavity	7·7	2·0	5·7	5
Baby mice	Intracerebral	8·2	3·2	5·0	14
	Intraperitoneal	8·2	1·8	6·4	14
	Intracardiac	8·4	2·0	6·4	14

± Titres are expressed as the logarithm to the base 10 of the endpoint dilution.

* Ratio of virus titre to "RNA" titre.

For any route of inoculation, the virus titre is greater than the titre of "RNA". The difference between virus and "RNA" is usefully expressed as the logarithm of the ratio of virus titre to "RNA" titre (V/R ratio). In the chick embryo this ratio is smallest for the chorioallantoic route of inoculation, and in the baby mouse it is smallest after intracerebral inoculation. The highest absolute titre of "RNA" was obtained on the chorioallantois.

Because inoculation of the choriollantoic membrane seemed to be the best method for titration of "RNA", variations of this technique were further examined.

Titration of "RNA" on the chorioallantoic membrane.

Time interval between dropping the membrane and inoculation. In the standard titration procedure, the membrane was dropped with 0·05 ml. of the inoculum. Where it was required to drop the membrane before inoculation, this was done with 0·05 ml. of saline. The inoculum of 0·05 ml. was later squirted in so that it splashed over the dropped chorioallantoic membrane. Three experiments yielded the following values for the titre of "RNA": dropping with inoculum, 3·9, 4·5 and 4·2 log; dropping membrane 30 minutes before inoculation, 1·0, 2·2 and 1·5 log; dropping membrane 24 hours before inoculation, 2·7, 3·0 and 2·9 log.

It seems clear that for highest titres, the membrane should be dropped with the inoculum. It may be supposed that dropping of the membrane is mildly traumatic and that this trauma causes the slow appearance of substances inimical to "RNA" – perhaps serum. The partial recovery of susceptibility after 24 hours is not due to the ageing of the egg, but may be due to the resorption of serum.

Time interval between drilling eggs and dropping the membrane. In these experiments, the membrane was dropped with the inoculum at various times after the shells had been drilled with a dental drill. In two series of tests there

was a slight apparent fall in titre of the "RNA" samples when the interval between drilling and inoculation was prolonged. The figures were as follows: in the first series, 1-minute interval, 4·6 log; 12-minute interval, 3·8 log; 45-minute interval, 4·0 log; in the second series, 5-minute interval, 5·0 log; 15-minute interval, 4·8 log; 50-minute interval, 4·3 log. It is doubtful whether this fall is significant; but as a routine we have inoculated eggs as soon as possible after drilling, and generally this has been within 10 minutes.

Age of eggs. One sample of "RNA" was titrated by inoculation onto the chorioallantoic membrane of eggs aged 10, 11, 12 and 13 days. Within experimental error, the titre did not vary between groups. We have routinely used eggs 12 days of age for inoculation.

Time of incubation of eggs after inoculation. Inoculation of a moderate number of infective doses of "RNA" did not kill eggs within two days. The majority of expected deaths had occurred by 72 hours, but there were a few deaths between three and four days. This is reflected in the LD_{50} titre of "RNA" or virus, estimated by death at three days or at four days. In two typical experiments, the titre of "RNA" at three days was 3·8 and 4·2 log and at four days, 5·0 and 4·6 log respectively. The titres of two samples of virus at three days were 6·9 and 7·4 log and at four days 7·8 and 7·8 log respectively.

It was also possible to estimate the infective titre of either "RNA" or virus by counting pocks present on the chorioallantoic membrane 48 hours after inoculation. The titres of the above samples, as judged by the pock count at two days, were approximately the same as the lethal endpoint estimated at three days, viz. 4·0 log for "RNA" as compared to 4·2 log, and 7·3 log for virus as compared to 7·4 log.

General procedure. The method of choice has been to titrate infectivity of virus or "RNA" by inoculation of the chorioallantoic membrane of 12-day-old chick embryos. The lethal endpoint has been estimated at three days, when specific deaths produced characteristic changes in the embryo and its membrane, rather than at four days when autolysis had confused this picture in many eggs.

Stability studies.

Fresh virus was diluted $10^{-2.5}$ in 10 p.c. NRS; "RNA" was made in phosphate saline pH 7·7 and used undiluted. Each preparation had an initial infective titre of 4·8 log. Aliquots of each were held at various temperatures for 30 minutes and then titrated. Fig. 1 shows that "RNA" and virus infectivities behaved similarly; the estimated 1 p.c. thermal endpoints were about 48° C.

These determinations were made, using different diluents for virus and "RNA". Huppert and Sanders (1958) have pointed out that it is sometimes difficult to prepare virus and "RNA" under the same conditions, so that they may be suitably compared. This is especially so in the comparison of their

heat stabilities. In an effort to meet this requirement we used as the diluent for virus (diluted $10^{-2.5}$) a mixture of equal parts of a 20 p.c. normal adult mouse brain extract and undiluted "RNA" made from normal baby mouse brains. Correspondingly, the infective "RNA" was mixed undiluted with an equal volume of 20 p.c. normal adult mouse brain extract. Heated in this diluent, the 1 p.c. survival endpoint of "RNA" was 40° C. and of virus 39° C.

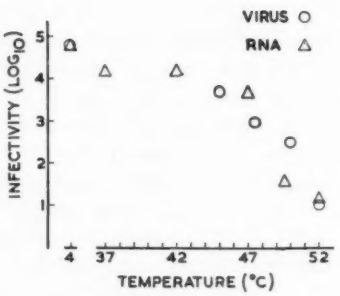

Fig. 1. Infective titre of MVE virus or "RNA" after being held at various temperatures for 30 minutes.

During the past year a small proportion of samples of "RNA" have been found to be less stable than the above figures would suggest. For example, some batches lost 3 log of infectivity after standing for 30 minutes at 20° C. Other batches had intermediate stabilities. This decreased stability may be due to components in the preparations other than "RNA". Two experiments would suggest that RNAse derived from the original brain tissue may survive phenol treatment and be at least partly responsible. In the first experiment, crystalline RNAse was dissolved in a 20 p.c. (w/v) normal baby mouse brain extract to give a final concentration of 50 μg/ml. This mixture was then treated three times with phenol as in the standard procedure for RNA preparation. RNAse activity was estimated in the manner described later in this paper. The titre of RNAse in the original mixture was 5·2 log; after the first phenol treatment it was 2·4 log, after the second phenol treatment 1·2 log, and after the third phenol treatment, 0·5 log. In other words, the final product could be diluted threefold and still inactivate 100 LD_{50} of infective "RNA" upon incubation at 20° C. for 30 minutes. In the second experiment, we determined the heat stability of "RNA" prepared by one, three and five phenol treatments. The infectivity titres and the thermal endpoints of the "RNA" preparations are given in Table 2. "RNA" recovered after one exposure of virus to phenol was much less stable than "RNA" recovered after three or five exposures to phenol. After one exposure to phenol, the "RNA" lost 99 p.c. of its original infectivity (4·2

TABLE 2.

Thermal stability of "RNA" prepared by different numbers of phenol treatments.

Number of phenol treatments	Original titre of RNA	1% survival* endpoint
1 (6 minutes)	4·2 log	25° C.
3 (6+2+2 minutes)	4·3 log	46° C.
5 (6+2+2+2+2 minutes)	4·5 log	44° C.

* Temperature at which 1 p.c. of infectivity remained after 30 minutes.

log) at 25° C. for 30 minutes. After either three or five exposures to phenol, "RNA" retained 1 p.c. of its titre at 46° C. for 30 minutes.

Freezing and thawing. One cycle of freezing and thawing did not reduce the infective titre of virus prepared as a 20 p.c. suspension of brain in phosphate citrate saline, nor of the "RNA" freshly prepared from this by phenol.

In general, for the work reported in this paper, we have used either fresh "RNA" or "RNA" kept frozen at −70° C. for a few days. Some observations of the thermal stability of "RNA" stored at −70° C. for longer than eleven days have suggested that such "RNA" has a lower thermal stability.

Inhibition of "RNA".

Inhibition by serum. Normal rabbit serum inhibited or destroyed the infectivity of "RNA". The inhibition was readily titrated as follows. Falling dilutions of serum were prepared in phosphate citrate saline and mixed at 4° C. with an equal volume of "RNA" containing 200 infective doses per 0·05 ml. The mixture was held at 4° C. for 30 minutes and then 0·05 ml. was inoculated into each of six eggs. The final dilution of serum which inhibited infectivity in this mixture was taken as the titre of inhibitor in the serum. This titre was about 500 in most samples of normal rabbit serum tested but varied between 300 and 10,000. Serum inhibitor of about this titre was found in all the species studied, namely, human, mouse, guinea-pig and chick embryo. The presence of this inhibitor has so far prevented effective serological examination of preparations of "RNA".

The heat stability of the inhibitor in normal rabbit serum was estimated by holding a 1 in 10 dilution of serum in normal saline, at various temperatures for 15 minutes and then titrating as above. In a typical experiment, inhibitor in unheated serum had a titre of 500. Heating at 37° C. did not affect the titre. After holding at 56° C., the titre was 600; after holding at 62° C., the titre had fallen to 20, and was less than 10 after holding at 65° C. or 100° C. The thermolability of serum inhibitor at first suggested that it was not classical ribonuclease. Pancreatic ribonuclease did not lose activity when diluted in phosphate citrate saline and held at 100° C. for 15 minutes. However, when ribonuclease was diluted to 0·1 μg/ml. in 10 p.c. NRS, and then heated for 15 minutes, its activity was destroyed at 100° C. but not at 65° C.

The serum inhibitor of "RNA" was not deposited by centrifugation at 93,000 *g* for 90 minutes, but was recovered quantitatively in the supernatant.

The serum inhibitor of MVE "RNA" is possibly the same as that described by Colter, Bird and Brown (1957) and Alexander *et al.* (1958) as inhibiting "RNA" derived from crude preparation of other viruses, and described by the latter author as probably a ribonuclease.

Inhibition by tissue extracts. Extracts (20 p.c. wet weight/vol.) were made in phosphate citrate saline, from spleen, lung, liver, brain and kidney of normal adult mice. Extracts from liver, kidney and brain did not inhibit "RNA" infec-

tivity, but extracts of spleen and lung contained an inhibitor which was active under the same conditions as the inhibitor in normal rabbit serum. Such an extract of spleen had a titre of 35,000 in terms of original splenic tissue, while the lung extract titred at 4,000. The splenic inhibitor was recovered quantitatively in the supernatant after centrifugation at 93,000 *g* for 30 minutes. The inhibitor in the crude splenic extract could be completely destroyed by standard phenol treatment, as used for the production of "RNA".

To investigate the possibility that the serum inhibitor might be produced and secreted by the spleen, two experiments were undertaken. In the first, serum and spleen were taken from the one mouse and tested for inhibitor. Per unit volume, serum contained 3000 units of inhibitor and spleen 40,000 units. In the second experiment, three mice were bled and their sera stored. They were then splenectomized and allowed to convalesce for 17 days. They were again bled. The inhibitory titre of the serum did not decrease during this interval, but stayed in the range 10,000-12,000 for each mouse.

Inhibition by ribonuclease (RNAse). On the assumption that any effect of RNAse on preparations of "RNA" would be due to an enzymatic action, the following procedure was adopted to titrate the activity of this enzyme. Falling dilutions of RNAse were made in phosphate citrate saline and each was mixed with an equal volume of "RNA" containing 200 infective doses per 0·05 ml. The mixtures were held at 20° C. for 30 minutes and each was then inoculated into one lot of eggs. The reciprocal of that final dilution of RNAse which allowed the "RNA" to infect 50 p.c. of the eggs was taken as the titre of RNAse activity. The control to this experiment involved holding the "RNA" dilution (without RNAse) at 20° C. for 30 minutes and then titrating its infectivity, which was required to be approximately 2 log. In three experiments the titre of RNAse was 0·0003, 0·0007 and 0·002 μg/ml. As described later, RNAse is appreciably less active against "RNA" in the presence of an aqueous extract of adult mouse brain. Under these conditions, the average of the titration endpoints of RNAse in two experiments was 0·07 μg/ml.

Lack of inhibition of virus. The infectivity of virus preparations was not reduced by the above reagents which had been found to inhibit "RNA". Normal rabbit serum is used as a routine diluent for virus and is presumed not to inactivate the virus. To estimate the reaction of the other reagents with virus, virus was diluted 10^{-5} in 10 p.c. normal rabbit serum to give a suspension with an infectivity endpoint approximately 2 log. The suspension was held at 4° C. for 30 minutes with extracts of mouse spleen, liver or lung and then inoculated into eggs. At concentrations up to 1:10 of the tissue, the extracts were found not to inhibit virus infectivity. As a refinement of this, the infectivity of the mixture containing 1:10 splenic extract and 10^{-5} of virus was titrated. It contained 10^2 units of infectivity which indicated that there had been no inhibition of the original virus infectivity.

RNAse did not inactivate virus. This was determined in one experiment

by diluting virus 10^{-4} in 10 p.c. adult mouse brain extract and adding an equal volume of RNAse containing 1 mg./ml. The mixture was held at 20° C. for 20 minutes. The titre of virus in this mixture was found to be 2·0 log compared with 1·7 log in the control without RNAse. In four other experiments using undiluted crude virus, prepared as a 1 in 5 suspension of infected mouse brain, RNAse (up to 500 μg/ml.) at 20° C. for 30 minutes did not decrease the titre of the virus. In one further experiment under the same conditions, a virus preparation, diluted $10^{-4.5}$ in adult mouse brain extract, to contain 100 LD_{50} did not lose infectivity in the presence of 400 μg/ml. of RNAse.

Protection of "RNA".

Extracts of adult mouse brain and liver were found to prevent the destruction of "RNA" infectivity by normal rabbit serum, splenic extract or RNAse. To demonstrate the effect against serum inhibitor, falling dilutions of adult mouse brain extract were mixed with a standard dilution of serum and then a calculated 100 LD_{50} of "RNA" was added. The mixtures stood at 4° C. for 30 minutes and were then inoculated into eggs. The endpoint was taken as that dilution of adult mouse brain extract which would allow the survival of 1 LD_{50} of "RNA". Against 10 inhibitory doses of serum (1 in 50 dilution of our standard normal rabbit serum), the titre of protecting agent in adult mouse brain was 30.

The demonstration of protection by brain extract required a certain order of mixing of the reagents. For example, when adult mouse brain extract dilutions were mixed with "RNA", held at 4° C. for 10 minutes, then mixed with 1:50 normal rabbit serum and again held at 4° C. for 30 minutes the endpoint of protection by the brain extract was still at 1 in 30. However, when "RNA" was mixed with normal rabbit serum in the above proportions, held at 4° C. for either two or ten minutes and then mixed with dilutions of adult mouse brain extract, no protection was demonstrated even by concentrations of adult mouse brain extract as high as 1 in 5.

Using the above method of titration, a protective agent was found to about the same titre in extracts of brains of guinea-pigs and sheep. The protective agents in the brains of all three species were stable to freezing and thawing. The titre of protective agent in mouse liver, when estimated in a similar fashion, was found to be 150 and in extracts of mouse kidney, 75.

Mouse liver extracts would also protect "RNA" against the inhibitor in mouse spleen; this hepatic protecting agent was estimated in the same way as that in the brain extract. Against a 1 in 500 dilution of spleen (i.e. 60 inhibiting doses), the titre of protective agent in liver was 30 but in brain was less than 5.

To demonstrate the protecting effect of adult mouse brain extract against RNAse, a different technique was used. Falling dilutions of RNAse were made in phosphate citrate saline and each dilution was mixed with a preparation of "RNA" in adult mouse brain extracts so that the final mixture contained a calculated 2 log of infective "RNA" per 0·05 ml. The mixture was held at 20° C.

for 30 minutes. The endpoint of RNAse action was considered to be the dilution which would allow the survival of 1 LD_{50} of "RNA". In two experiments, the endpoints were reached with 0·02 and 0·2 μg/ml. respectively of RNAse in the final mixture. This is a 100-fold greater concentration of RNAse than was present at the endpoint of RNAse activity found when phosphate citrate saline was the diluent for the system.

The protective agent in adult mouse brain is thermolabile. A 20 p.c. suspension of normal mouse brain in phosphate citrate saline was prepared. The titre of protective agent was 50 against a 1:50 dilution of standard normal rabbit serum. Samples of this brain extract were held undiluted at 20° C. or 37° C. for 75 minutes and in both cases the titre was then 60. After heating for 15 minutes at 56°, 65° or 100° C., the titre was less than 10.

A suspension of adult mouse brain was prepared as above and centrifuged at 93,000 *g* for 90 minutes. The titres of the original preparation and of the supernatant were both 30. The protecting agent in mouse liver was also recovered quantitatively in the supernatant after centrifugation at 93,000 *g* for 90 minutes.

Serology.

Infected chorioallantoic membranes were harvested from eggs which had been inoculated two days previously with 10 LD_{50} of "RNA". At this stage, the eggs were alive and the membranes contained a high titre of infective agent. The membranes were ground and suspended in 10 p.c. (v/v) normal rabbit serum and briefly centrifuged; the supernatant was removed and injected intraperitoneally into baby mice. The mice became sick four days later. The brains were harvested, suspended in 10 p.c. normal rabbit serum and titrated by the intraperitoneal route in suckling mice. The titre of this material was 7·8 log. This material was titrated against standard MVE immune guinea-pig serum by the same route. The serum had a neutralizing titre of 1:256 against 2 log of both standard MVE virus and the infective agent derived from "RNA", a result which suggests that the virus derived from cells infected with "RNA" was identical with the virus from which the "RNA" was derived.

DISCUSSION.

Treatment of crude preparations of MVE virus with phenol yielded an infective product which differed from the original virus in several ways. Most importantly the infectivity was rapidly destroyed by very low concentrations of crystalline pancreatic ribonuclease, and by dilute serum. The evidence is highly suggestive that this infective product is essentially ribonucleic acid, and we have referred to it as "RNA"; but there is yet no evidence as to whether the infective product is composed only of ribonucleic acid. Other substances are certainly present in the preparation of "RNA" and some of these may be directly associated with the ribonucleic acid.

One purpose of this paper is to report techniques. The chick embryo has proved a very satisfactory host in which to detect infective "RNA" of MVE virus. Titration endpoints of fresh "RNA", or crude virus, were sharp and repeatable. The titre of "RNA" determined in the chick embryo was higher than the corresponding titre in the suckling mouse brain, and this was considered an advantage although the titre of crude virus was lower in the chick embryo than in mice. Also, the chick chorioallantois may prove more suitable than mice for later experiments on the dynamics of infection by "RNA".

The inactivation of infective "RNA" provides a very sensitive and simple method for the titration of the enzymic activity of ribonuclease. For example, 100 chick embryo LD_{50} of MVE "RNA" was destroyed by 0·0007 μg/ml. of ribonuclease in 30 minutes at 20° C. This titration technique has been used in the present work.

Several results will be discussed briefly. The relative thermostability of carefully prepared "RNA" was unexpected. The close correspondence between the thermal inactivation curves of virus and "RNA" is of some interest, and a first thought might be that thermal inactivation of virus is due entirely to damage to the ribonucleic acid. However, the recovery of viable "RNA" from heat inactivated virus is evidence against this view (Ada and Anderson, 1959).

It is a point of some practical significance that MVE "RNA" is rapidly destroyed when diluted in serum and that virus rapidly loses 3 to 5 log of infectivity when kept in phosphate citrate saline at 20° C. This, in fact, often allows a clear distinction to be made between infectivity due to virus and that due to "RNA" in any one preparation.

The inhibition or destruction of infective "RNA" by serum has been reported for other viruses, and this effect of serum has made it difficult to examine infective "RNA" by serological technique. Gamma globulin appears to be free of this serum inhibitor at least in the case of poliomyelitis virus "RNA" (Alexander *et al.*, 1958).

The possibility that the serum inhibitor of the infectivity of "RNA" is in fact a ribonuclease is suggested by two findings. The first is that ribonuclease has been detected in serum by independent techniques (Métais and Mandel, 1955). The second is that the thermolabile agent in extracts of mouse brain or liver interfered with both serum inhibitor and crystalline ribonuclease in their action on infective "RNA". On the other hand, the thermostability of serum inhibitor differed from that of crystalline ribonuclease. Serum inhibitor diluted in saline was destroyed at about 62° C. In phosphate citrate saline pancreatic ribonuclease was stable at 100° C., but in 10 p.c. normal rabbit serum it was destroyed at 100° C., though stable at 65° C.

The survival of a remnant of added pancreatic ribonuclease during the standard phenol treatment requires comment. This treatment consisted of three exposures to phenol, and with each exposure there was a progressive reduction in activity of the ribonuclease; but even after three exposures some

activity remained. This result may be relevant to the following situations:

(i) If crude virus is treated with pancreatic ribonuclease, and then used as a source of infective "RNA", the yield of "RNA" may be reduced by the remaining ribonuclease activity. This is the type of experiment which might be used to determine whether "RNA" comes from intact virus particles.

(ii) It was found that "RNA" which had been produced by exposing crude virus to only one lot of phenol was less stable than the routine "RNA" produced by three such exposures. A reasonable hypothesis would be that crude virus prepared from mouse brain contains ribonuclease, although in this situation the enzyme would be masked by the protective agent derived from brain. It has also been shown that the protective agent is destroyed by phenol. It is therefore suggested that during the first exposure of virus to phenol both ribonuclease and "RNA" are released in the one solution. Such a mixture would appear as a solution of relatively unstable "RNA".

(iii) In the standard phenol treatment, if brain ribonuclease occasionally persisted through three exposures to phenol, it might account for the rare sample of "RNA" with a thermal stability which was lower than normal.

(iv) If the above assumptions are correct, it seems inevitable that there should be some destruction of "RNA" during the course of the standard phenol treatment used to prepare "RNA" from virus. It is a matter of conjecture as to how far this was responsible for the fact that "RNA" titres were always much lower than the titres of the original crude virus.

These considerations suggest that one necessary condition for the preparation of stable infective "RNA" from a virus is that any ribonuclease in the original virus suspension should be inactivated. In the case of MVE derived from baby mouse brain, this inactivation of RNAse seems to be just barely accomplished by the standard phenol treatment. This prompts the thought that with other RNA viruses, grown in brain or other tissues, the currently used procedures may not always fulfil this condition, and that surviving tissue ribonuclease may prevent recognition of infective "RNA" which has been isolated.

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STUDIES OF ARTHROPOD-BORNE VIRUS INFECTIONS IN QUEENSLAND

I. A SEROLOGICAL SURVEY OF ABORIGINAL MISSIONS BORDERING THE GULF OF CARPENTARIA

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SUMMARY.

Of 267 sera collected from aborigines at missions or towns in Queensland bordering the Gulf of Carpentaria, 145 had neutralizing antibodies to Murray Valley encephalitis. The immunity rate was highest at Mitchell River, Aurukun and Mapoon, and lowest at Weipa.

Studies of young children and fowls at Mitchell River suggested that the last dissemination of MVE virus occurred there in early 1954 or, at latest, early 1955.

The haemagglutination-inhibition test was found to give a good approximation to the more expensive neutralization test. Small numbers of sera neutralized Japanese B encephalitis, West Nile virus, dengue 1, or dengue 2, but it is considered that these could be explained by immunological overlap, and that MVE immunity is the dominant feature of the antibody pattern demonstrated.

INTRODUCTION.

Ludford and Cook (1957) presented evidence that Murray Valley encephalitis virus (MVE) infection was widespread in north Queensland in 1951, and that the virus returned or persisted in the Gilbert and Einasleigh River areas at least as late as 1954. They demonstrated complement fixing antibodies to MVE in the serum of patients convalescent from dengue, making it evident that future studies of MVE in Queensland would need to take account of dengue. The present investigations were planned to extend previous observations on the geographical distribution of MVE infection, to define the time of the most recent dissemination of virus, to evaluate the haemagglutination-inhibition (HI) test as a survey tool, and to determine whether other group B viruses (Casals and Brown, 1954) might occur in the area.

MATERIALS AND METHODS.

Collection of sera.

Aboriginal missions at Mapoon, Weipa, Aurukun, Edward River, Mitchell River, Doomadgee and Mornington Island, and the towns of Burketown and Normanton (Fig. 1) were

Fig. 1. *Towns and missions included in the survey.*

visited in November and December, 1957, on clinic flights of the Royal Flying Doctor Service. Two hundred and seven blood samples were collected from children and young adolescents who had spent their lives in that area, or from adults whose movements were known. Blood was collected in 15 ml. "venules" (Bayer), and sent by air to Brisbane at the conclusion of the flight. The serum was then separated and stored at — 20° C. The time interval between collection and storage of serum was from three to seven days.

A further visit was made to Mitchell River Mission in June-July, 1958, and 107 blood samples collected from young children, adults, fowls of known age, and some wild birds and mammals. This mission was chosen for more detailed study, because of the high MVE immunity rate found in the older children, and because it had a weekly commercial air service, and was therefore comparatively accessible for future studies. Sera were separated within 24 hours of collection, stored at 4° C. until taken by air to Brisbane, and afterwards at — 20° C.

Virus strains.

The strain, origin, and previous history of each virus used were:

MVE, maintained through an unknown number of passages in this laboratory since receipt in March, 1952, from Walter and Eliza Hall Institute, Melbourne.

Dengue 1, strain Hawaii, from American Type Culture Collection, Washington, was used at the 116th mouse passage.

Dengue 2, strain New Guinea C, from Dr. W. McD. Hammon, University of Pittsburgh, was used at the 24th mouse passage.

Japanese B encephalitis (JBE), strain Nakayama, from American Type Culture Collection, Washington, was used at the 42nd mouse passage.

West Nile virus (WN), strain Sarafend, from Department of Microbiology, Harvard School of Public Health, was used after an unkown number of mouse passages, six passages in human tissue culture, and two passages in suckling mice.

Pools of infected mouse brain (20 p.c. in normal inactivated rabbit serum diluted 1:10 in saline) were stored in sealed ampoules at — 60° C., in a dry ice cabinet or an electric "Revco" refrigerator. Pools of MVE, JBE and WN had titres of 10^8 to 10^9 mouse LD_{50} per 0·03 ml. inoculum. Dengue 1 and dengue 2 gaves titres of only 10^5 to 10^6 LD_{50} per 0·03 ml.

Standard antisera.

Antisera against MVE, JBE and WN were obtained from guinea pigs inoculated intraperitoneally with virus pools prepared from infected chick embryos. Some MVE antisera were prepared by intracerebral inoculation of guinea-pigs. Dengue 1 and dengue 2 antisera were obtained from adult mice inoculated with three doses of infected mouse brain intraperitoneally, according to the method of Casals (1945).

Neutralization tests.

The sera were initially tested by the technique previously in use at this Institute (Ludford and Cook, 1957, second method described), in which virus-serum mixtures were incubated at 4° C. for 30 minutes before inoculation. Discrepancies between neutralization and HI results led to this technique being evaluated by determining neutralization indices on a number of sera. It was found that incubation of virus-serum mixtures at 37° C. for 60 minutes gave indices tenfold higher, without lowering the virus titre. The final technique used therefore was to incubate undiluted serum (inactivated at 56° C. for 30 minutes) with an equal amount of virus dilution (calculated from prior titration to give 100 LD_{50} in the 0·03 ml. final inoculum) for 60 minutes at 37° C. Mixtures were then placed in an ice bath until inoculated into mice.

Six mice were used for each serum. MVE tests were done by intraperitoneal inoculation into five- to eight-day-old mice, other virus tests by intracerebral inoculation into 21-day-old mice. Virus titrations were set up with each test to determine the actual amount of virus used. Mice were observed for 14 days (MVE, JBE and WN), or 21 days (dengue 1 and dengue 2). Sera were considered positive when four or more mice survived.

Haemagglutination-inhibition tests.

MVE haemagglutinin was prepared and HI tests performed according to the methods described by Clarke and Casals (1955, 1958), and Casals and Brown (1954), with certain modifications. Thus, haemagglutinin was stored in sealed glass ampoules at — 60° C. for periods up to a year without fall of the initial titre of 1:5120. Antigen titrations and HI tests were carried out in plastic plates, with final incubation at 37° C. at pH 6·6.

Nonspecific inhibitors were removed from sera by treatment with kaolin (British Drug Houses, London; endorsed "Society of Leather Trades Chemists' specifications") or by treatment with acetone and ether (Porterfield, 1954). Early tests were done with red cells from day-old chicks, and preliminary absorption of the sera was not necessary. Later, goose cells were used (Porterfield, 1957); agglutinins to them were removed by adding one volume of 10 p.c. cells in pH 9 buffer to 20 volumes of 1:10 serum, incubating the mixture at 37° C. for 30 minutes, and then centrifuging it.

Twofold dilutions of absorbed sera from 1:10 to 1:640 in pH 9 borate-buffer were incubated with four to eight units of antigen for one hour at 4° C., before the goose cells were added. The highest dilution showing no agglutination was read as the end point.

RESULTS.

Neutralization tests. The results of neutralization tests on the sera collected in 1957 are summarized in Table 1. One hundred and twenty-four of 206 sera neutralized MVE. A small group of adults, bled at Weipa, but newly arrived from Hope Vale, near Cooktown, had no antibodies to MVE, but otherwise all the places visited had evidence of MVE infection. The proportion of sera with antibodies was of the order of 80 p.c., except at Weipa (10 p.c.) and Mornington Island (under 50 p.c.).

TABLE 1.

Results of neutralization and HI tests on 207 sera collected November-December 1957.

Area	Number of sera	Age distribution	HI test (MVE)	Neutralization tests					Percentage with antibodies*
				MVE	JBE	WN	dengue 1	dengue 2	
Mapoon	23	9-16	17/23	17/23	13/20	3/21	3/22	5/23	87
Weipa	21	12-17	5/19	2/21	3/19	0/16	0/21	1/20	29
Aurukun	30	13-16	23/30	25/30	14/26	2/29	3/29	3/30	87
Edward River	10	15-35	5/8	6/10	1/3	0/3	0/7	0/5	60
Mitchell River	26	11-15	19/26	20/26	3/25	3/24	2/26	1/25	92
Normanton	10	8-12	5/10	8/10	2/9	1/6	0/10	0/6	80
Burketown	13	4-13	8/13	9/13	4/12	0/12	1/13	0/13	69
Doomadgee	27	7-12	13/27	18/26	5/21	1/21	2/26	0/24	78
Mornington Island	41	4-14	11/41	19/41	7/37	1/36	1/39	0/37	53
Hope Vale	6	18-35	1/6	0/6	0/4	0/4	3/6	4/5	83
Totals	207		107/203	124/206	52/176	11/173	15/199	14/188	

* Percentage positive to one or more tests.

TABLE 2.

Sera from Mitchell River Mission according to age.

Age	HI MVE	Neutralization tests					Percentage positive to one or more tests
		MVE	JBE	WN	dengue 1	dengue 2	
2-3	0/15	0/15	0/13	0/11	0/14	0/13	0
4	5/9	4/9	1/7	0/7	0/7	0/7	67
5-6	1/8	1/8	0/7	0/7	0/7	0/7	12
*11-15	19/26	20/26	3/25	3/24	2/26	1/25	92
16-20	6/7	6/7	4/7	1/7	0/7	1/7	86
21-30	5/8	2/8	0/8	0/8	2/8	1/8	62
31-40	6/7	4/7	1/7	2/7	0/7	1/7	86
>41	4/7	4/7	4/7	0/7	1/7	1/7	86
Totals	46/87	41/87	13/80	6/78	5/83	5/81	

* The 11-15 year group were collected in November, 1957, the others in June-July 1958.

Neutralization tests on human sera collected in 1958 are summarized in Table 2. In the two groups, 267 sera were tested. One neutralized all five viruses, four neutralized four viruses, 14 neutralized three, and 48 neutralized two viruses. Sixty-four of these 67 sera neutralized MVE, 53 neutralized JBE, 15 dengue 1, 14 dengue 2, and 13 WN. In addition, 81 sera neutralized MVE alone, nine JBE, three dengue 1, four dengue 2 and one WN. There were thus 20 sera which did not neutralize MVE, but did neutralize one or more of the other viruses. Five of these came from the Hope Vale group; they neutralized dengue 1 or dengue 2, or both, and presumably indicate prior infection with dengue.

Haemagglutination-inhibition tests. The sera collected in 1957 were tested for HI antibodies to MVE after removal of nonspecific inhibitors with kaolin. At about that time, a group of stored human sera, not included in the present series, was tested with results that suggested that nonspecific inhibitors were not completely removed by kaolin. The north Queensland sera were therefore retested after acetone-ether treatment. The titres obtained by the two methods showed an obvious close correlation (coefficient of correlation + 0·73), but the kaolin titre was usually one to two dilutions higher than the acetone titre. Retesting with kaolin gave identical titres to the first test, suggesting that the lower acetone titre was not merely due to the period of storage, or to repeated thawing and freezing. The Rockefeller Foundation reports (Clarke and Casals, 1958) specify a kaolin that was not available here; it is possible that the kaolin used did not completely remove nonspecific inhibitors. Whatever the cause, it was decided to use acetone-ether extraction as the standard method, and all the 1958 sera were tested in that way. A titre of 1:10, after acetone treatment, was accepted as significant. In a few cases, in which amount of serum did not permit a serum tested with kaolin to be repeated with acetone, a kaolin titre of 1:40 was accepted as significant.

The distribution of HI antibodies at the missions is shown in Table 1, and the age distribution at Mitchell River in Table 2. The neutralization and HI tests results on human and animal sera are correlated in Table 3. It is evident

TABLE 3.

Correlation between HI and neutralization test results on 279 sera.

		Neutralization Test		
		+*	± or −	
HI Test	†+	132	14	146
	−	44	89	133
		176	103	279

*+ = neutralizing one or more of the viruses tested.
†+ = HI titre $<$ 1:10.

that the tests agree in 221 of 279, or 80 p.c. Forty-four sera had neutralizing antibodies without HI antibodies, and 14 sera had HI antibodies in low titre, but did not neutralize any of the five viruses. Six of these, however, showed some evidence of protection in that three of six mice survived in tests against MVE (4), dengue 2 (1), WN (1) or JBE (1). Sera collected seven months later from two of these patients had similar HI titres, but did neutralize MVE.

The titre of HI antibodies and the presence of detectable neutralizing antibodies showed a close relationship. Thus 33 p.c. of sera with negative HI tests had neutralizing antibodies, but all sera with HI titres of 1:80 to 1:640 neutralized at least one virus. The geometric mean HI titre of the four sera that neutralized four viruses was 1:190, of the 14 sera that neutralized three viruses 1:53, and of the 48 sera that neutralized two viruses 1:23.

Age incidence of antibodies. Neutralization and HI test results on all sera from Mitchell River Mission are tabulated by age in Table 2. It is evident that children aged two or three had no evidence of previous infection, whereas a number four years old had neutralizing antibodies. The youngest child with antibodies was born in April, 1954. Adults over 20 had a lower rate of neutralizing antibodies than the 11-20 age group ($P < 0{\cdot}02$), but the proportions with HI antibodies did not differ significantly. Similar age profiles were not obtained at other missions. Serum was, however, obtained at Mornington Island from 11 children born after the 1951 outbreak of illness there (Ludford and Cook, 1957). One neutralized MVE, and another neutralized JBE, but was equivocal to MVE.

Tests of animal sera. A small number of bird and mammal sera were obtained at Mitchell River Mission. Eleven fowls introduced in August, 1957 (ten months before they were bled) had no HI or neutralizing antibodies to MVE, but six of seven fowls introduced in July, 1953, had antibodies, in three cases to both tests. All of six wallabies (*Macropus* spp.) had antibodies, four being positive to both tests. Serum from one of two plain turkeys (*Eupodotis australis* Gray) neutralized MVE, JBE and dengue 1, and had a HI titre of 1:640. One of five wild ducks (*Tadorna radjah* Garnot) neutralized dengue 1 and dengue 2, but not MVE. It was negative to the HI test. Serum from one galah (*Kakatoë roseicapilla*) neutralized MVE but was negative by HI test.

DISCUSSION.

It is evident that antibodies to MVE can be demonstrated in a high proportion of children and adolescents in the area of Queensland bordering the Gulf of Carpentaria. Observed variations in this proportion at different places must be interpreted cautiously, as the groups are small and vary a little in age composition. The low rate at Weipa does, however, appear to be significantly different from that at the missions to its immediate north and south.

The present studies add support to the statement of Ludford and Cook that there was dissemination of MVE as late as 1954. Children born between July,

1953, and April, 1954, and fowls introduced into the area in July, 1953, had developed antibodies by June, 1958. Children born after April, 1954, and a group of fowls introduced in August, 1957, had no antibodies.

Infants born in an area with a high rate of neutralizing antibodies may have placentally transmitted passive immunity for part at least of the first year of life. It is difficult to estimate how soon after birth such children would become susceptible to infection. Thus, while these findings suggest that the most recent spread of MVE at Mitchell River Mission occurred in the wet season of early 1954, it is possible that the spread occurred in early 1955. It seems unlikely that it was as late as the summer of 1955-56, when MVE infection was demonstrated in the Murray Valley (Anderson *et al.*, 1958) and in Papua (French *et al.*, 1957).

Haemagglutinin from MVE was prepared by Macdonald (1952), and studied by McLean (1956). The HI reaction was used by Anderson *et al.* (1958) in clinical investigation of patients, and by French *et al.* (1957) in characterizing an isolated virus. Beech *et al.* (1958) used the HI test in a serological survey of a mission in South Australia. Southam (1956) used the HI test in a survey of children in Japan for evidence of JBE infection. The present results suggest that the HI test will be a useful survey tool. It is cheaper and quicker to perform than the neutralization test, and the proportion of sera with HI antibodies gave a useful approximation to the neutralization antibody rate. Twenty-five percent of sera with neutralizing antibodies were negative to HI, a figure comparable to those found by Southam and Beech *et al.* in their surveys. These presumably represent old infections in which the HI titre had fallen. It should be pointed out, however, that the fall in the proportion of sera with neutralizing antibodies in the higher age groups at Mitchell River suggests that these antibodies too may disappear with time. Hammon *et al.* (1958) have observed this phenomenon with JBE antibodies on Guam.

The HI test is group specific (Casals and Brown, 1954). It was hoped that it would provide a useful screen test for any unknown group B virus in the area. The 14 sera with HI antibodies but no neutralizing antibodies are therefore of interest. The HI titres of these sera were low, and several gave equivocal results in one or more neutralization tests. The simple screening neutralization test employed, using a single dilution of virus, has statistical and technical sources of variability (Smith, 1957) which may give rise to errors in individual sera. In addition, the present tests, using inactivated sera without addition of labile factor (Morgan, 1945), may have classed as negative sera with minimal amounts of antibody. Beech *et al.* (1958) had a small number of HI-positive, neutralization-negative sera in South Australia, where dengue can be excluded, and the presence of a second group B virus seems less likely than in North Queensland. It would seem, therefore, that these 14 sera are not convincing evidence of the presence of an unknown group B virus in this area.

A similar problem is presented by the 20 sera that neutralized viruses other

than MVE. It is possible that they indicate previous infection with those viruses. On the other hand, previous surveys for group B virus antibodies have demonstrated that immunological cross-reactions occur between members of the group (Smithburn, 1954), and that a serum may neutralize one virus because of infection with one or more related viruses (e.g. the demonstration of yellow fever antibodies in Malaya by Smith, 1957). In the present series, the frequency of JBE antibodies could be explained by the close relationship between MVE and JBE. The ten sera that neutralized JBE alone may reflect the limitations of the techniques employed, rather than the presence of JBE infection in North Queensland. Five of the nine sera that neutralized dengue 1 or dengue 2, but not MVE, were from the small Hope Vale group, and probably represent dengue infection from outside the area under survey. While the present study does not completely exclude the possibility that dengue or JBE infections have occurred in the Gulf of Carpentaria area, it must be accepted that immunity to MVE is the dominant feature of the antibody pattern demonstrated, and that this pattern may well be due to MVE infection alone.

The high incidence of MVE antibodies is in contrast to the rarity of reported clinical encephalitis. Difficulties of diagnosis in this remote area, and a high proportion of subclinical infection in a small population, may both contribute to this. The introduction of susceptibles into the mining developments planned for this area may cause MVE to assume greater public health importance in the future.

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THE EFFECT OF ALLOXAN DIABETES ON THE METABOLIC ACTIVITY OF RAT MAMMARY GLAND TISSUE

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SUMMARY.

Alloxan diabetes results in loss of weight and considerable mortality in litters of lactating rats. Insulin replacement restores near normal litter growth rates and decreases litter death rate to zero.

Metabolism of rat mammary gland slices *in vitro* after alloxan treatment closely resembles that observed after hypophysectomy of lactating rats. Insulin replacement therapy *in vivo* corrects a biochemical lesion responsible for an abnormal production of lactic acid.

Diabetes renders mammary gland slices more sensitive to insulin *in vitro*, an opposite effect to that observed following hypophysectomy of lactating rats.

INTRODUCTION.

Despite the striking effects of insulin on the *in vitro* metabolism of lactating rat mammary gland tissue (Balmain, French and Folley, 1950; Balmain and Folley, 1951; Balmain, Folley and Glascock, 1954), the rôle of the pancreas, in control of mammary tissue metabolism, has been largely neglected, especially in the rat.

There is evidence that removal of the pituitary gland results in decreased blood insulin activity in the rat (Randle, 1955), also that pituitary removal results in severe curtailment of the metabolic activity of mammary gland tissue (Bradley and Cowie, 1956). Further, it has been shown that hypophysectomy of lactating rats severely depresses the ability of mammary gland tissue to respond to insulin (Bradley, 1958). The primary purpose of this investigation has been to ascertain whether such a lack of response to insulin could be the typical effect of decreased blood levels of insulin.

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METHODS.

All litters were reduced to eight at 2nd or 3rd day of lactation and were weighed daily.

Diabetic rats were prepared by the intravenous injection of a single dose of alloxan monohydrate (5 mg./100 gm. body weight) at the 7th-8th day of lactation. Estimations of blood sugar concentrations were made by a modification of the anthrone method (Prohkovnik and Nelson, 1953).

In the experiments involving measurement of litter weights, rats were allotted to one of the following groups:—

Group 1: Those not treated with alloxan (normal lactating group).

Group 2: Those receiving no further treatment after alloxan administration (alloxan-diabetic group).

Group 3: Those which received insulin replacement starting 24 hours after alloxan injection (insulin replacement group). Blood sugar estimations were made periodically on each rat of this group and were used to indicate the dosage of insulin to be given. Protamine zinc insulin was used and given subcutaneously once daily within the range of 1-4 units.

For all of the metabolic experiments rats treated with alloxan were maintained on insulin (as above), the groups being:

Group 4: No treatment (normal lactating group).

Group 5: Last injection of insulin 40 hours prior to the excision of the mammary gland tissue for *in vitro* testing (insulin deprived group).

Group 6: Last injection of insulin 16 hours prior to use of the tissue; otherwise treated in the same way as group 3 (insulin replacement group).

The experimental procedure, preparation of tissue for the *in vitro* incubation, measurements of glucose uptake and lactic acid production by the tissue slices, and estimation of significance of the results, were performed as described previously (Bradley, 1958).

RESULTS.

Litter weight measurements.

Litters of normal lactating rats gained weight at a constant rate over the period 8th-16th day of lactation.

Alloxan-diabetic rats were not able to maintain normal growth rates in their litters. Within 72 hours of alloxan administration the litter weights were no longer increasing and considerable mortality had occurred. Litter weights steadily decreased with time whilst the percentage mortality increased. The range of blood sugar values of the rats in this group was 450-700 mg. p.c. compared with a mean value of 105 mg. p.c. found in the normal lactating group.

Replacement therapy with insulin resulted in gains in litter weights at approximately the same rate as shown in normal rat litters. The mortality rate was reduced to zero. Milk was observed in the pups' stomachs within 12 hours after the first insulin injection. It was not possible to keep blood sugar values always in the normal range, thus blood sugar values in this group ranged from 40-370 mg. p.c. throughout the experiment period. However, even those rats

with a considerably higher than normal blood sugar content reared their litters satisfactorily provided that insulin injections were maintained. The results are shown in Fig. 1.

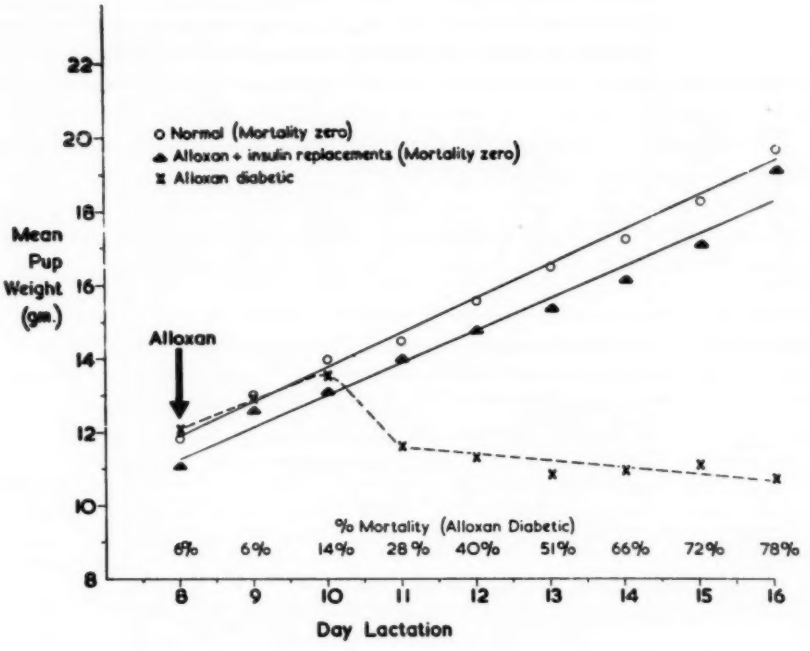
o Normal (Mortality zero)
▲ Alloxan + insulin replacements (Mortality zero)
x Alloxan diabetic
Mean Pup Weight (gm.)
Alloxan
% Mortality (Alloxan Diabetic)
6% 6% 14% 28% 40% 51% 66% 72% 78%
Day Lactation

Fig. 1. The effect of alloxan diabetes and insulin replacement on lactation.

Metabolic activity of tissue slices.

The series of experiments was designed to give two main comparisons:

(a) Comparison of the metabolic activities of the control slices (i.e. no *in vitro* treatment) in the three groups (4, 5 and 6); and

(b) comparison of the behaviour of tissue from the three groups of rats with respect to its response to insulin *in vitro*. The two insulin levels used were those previously found to be approximately the lower and upper limits of the dose response curve for the effect of insulin on normal lactating mammary gland tissue.

The results are incorporated in Tables 1, 2 and 3.

TABLE 1.

Mammary gland slices from normal lactating rats.

		Composite respiration[1]			Glucose uptake[2]			Lactic acid produced[3]		
		Control	Insulin μg./ml.		Control	Insulin μg./ml.		Control	Insulin μg./ml.	
Rat No.	Blood glucose mg. %		0·5	2·5		0·5	2·5		0·5	2·5
1	90	198+	203	251	1353	1417	1880	123	205	347
2	110	238	316	399	1717	2283	2853	157	345	640
3	110	221	238·	317	1527	1717	2127	78	92	185
4	110	220	280	308	1540	2093	2620	82	113	243
Means		219	259	319	1534	1877	2370	110	189	354
Variance ratio*		F=15·3; P<·01>·001			F=24·8; P<·01>·001			F=8·9; P<·05>·01		
"t" tests		Insulin μg./ml. 0·5 v. control t=2·2; P=·07 2·5 v. control t=5·5; P<·01>·001			Insulin μg./ml. 0·5 v. control t=2·9; P<·05>·02 2·5 v. control t=7·0; P<·001			Insulin μg./ml. 0·5 v. control t=1·3; P>·3 2·5 v. control t=4·1; P<·01>·001		

[1] μl.
[2] μg. / 100 mg. moist weight tissue/120 minutes.
[3] μg.

+ Each value is the mean of three replicate observations.

* Variance ratio (F) = $\frac{\text{mean square between treatments}}{\text{Interaction mean square (Rats} \times \text{treatments)}}$

(a) *Metabolic activity of control slices.* The metabolic activity of mammary gland slices from the normal lactating group of rats was similar to that previously described (Bradley and Cowie, 1956; Bradley, 1958). Large amounts of glucose were taken up by the slices, very little lactic acid was liberated into the medium and composite respiration values were high.

Slices from the insulin deprived group showed a considerably decreased ability to take up glucose from the incubation medium ($P < \cdot001$, "t" test comparison with normal tissue values). Simultaneously, there was a significantly increased lactic acid production ($P < \cdot02 > \cdot01$) and decreased composite respiration ($P < \cdot001$).

The control tissue from the insulin replacement group showed more variable behaviour. Tissue from rats in which reasonable control of blood glucose levels

was attained, i.e. approximating to those values found in normal lactating rats, were not obviously better with respect to the *in vitro* tests than those with higher or lower blood sugar values. With the exception of tissues from two of the rats used little effect of the insulin replacement therapy was observed on glucose uptake ($P > \cdot 2$ compared with insulin deprived group; $P < \cdot 05 > \cdot 01$ compared with normal group); however, there was a distinct decrease in the lactic acid production compared with insulin deprived group ($P < \cdot 01 > \cdot 001$) and no significant difference from the normal lactating group ($P > \cdot 7$). Composite respiration was extremely variable in the control tissue of the insulin replacement group and not significantly different from that of the insulin deprived group ($P > \cdot 3$); $P < \cdot 05 > \cdot 01$ compared with the normal group.

(b) *Responses to insulin* in vitro. The tissue from the normal group responded to the two insulin dose levels (see Table 1) in the same fashion as previously found with normal lactating mammary gland slices (Bradley, 1958). The *in vitro* insulin gave rise to: (i) increased glucose uptake with a graded

TABLE 2.

Mammary gland slices from alloxan diabetic rats.

		Composite respiration[1]			Glucose uptake[2]			Lactic acid produced[2]		
		Control	Insulin μg./ml.		Control	Insulin μg./ml.		Control	Insulin μg./ml.	
Rat No.	Blood glucose mg. %		0·5	2·5		0·5	2·5		0·5	2·5
1	700	62+	126	200	613	1107	1653	218	335	403
2	500	77	171	234	867	1533	1950	325	363	413
3	450	116	231	280	983	1833	2150	672	732	832
4	470	137	157	188	757	963	1307	718	800	1113
Means		98	171	226	805	1359	1765	483	557	690
Variance ratio*		F=20·74; P<·01>·001			F=34·2; P<·001			F=7·91; P<·05>·01		
"t" tests		Insulin μg./ml. 0·5 v. control t=3·67; P<·02>·01 2·5 v. control t=6·43; P<·001			Insulin 0·5 v. control t=4·6; P<·01>·001 2·5 v. control t=8·2; P<·001			Insulin 0·5 v. control t=1·40; P>·2 2·5 v. control t=3·92; P<·01>·001		

[1] μl.

[2] $\mu g. \Big/ 100$ mg. moist weight tissue/120 minutes

[3] μg.

\+ Each value is the mean of three replicate observations.

* Variable ratio (F) $= \dfrac{\text{mean square between treatments}}{\text{Interaction mean square (Rats} \times \text{treatments)}}$

TABLE 3.

Mammary gland slices from alloxan + insulin replacement treated rats.

		Composite respiration[1]			Glucose uptake[2]			Lactic acid produced[3]		
		Control	Insulin μg./ml.		Control	Insulin μg./ml.		Control	Insulin μg./ml.	
Rat No.	Blood glucose mg. %		0·5	2·5		0·5	2·5		0·5	2·5
1	40	136+	161	194	987	1290	1700	63	35	60
2	55	156	216	200	1013	1353	1187	225	205	178
3	85	223	268	255	1577	1910	1787	145	112	145
4	90	95	118	177	570	790	1167	82	78	77
5	190	41	94	153	607	1097	1517	68	67	157
6	220	107	179	243	903	1367	1953	75	52	225
7	370	159	259	335	1397	2120	2747	38	23	13
Means		131	185	223	1008	1418	1727	100	82	112
Variance ratio*		F=18·2; P<·001			F=17·7; P<·001			F=1·74; P>·2		
"t" tests		Insulin μg./ml. 0·5 v. control t=3·55; P<·01>·001 2·5 v. control t=6·05; P<·001			Insulin 0·5 v. control t=3·34; P<·01>·001 2·5 v. control t=5·86; P<·001			No significant insulin effect		

[1] μl. / [2] μg. / [3] μg. / 100 mg. moist weight tissue/120 minutes.

+ Each value is the mean of three replicate observations.

* Variance ratio (F) = $\frac{\text{mean square between treatments}}{\text{Interaction mean square (Rats} \times \text{treatments)}}$

effect from the lower to the higher insulin dose; (ii) a similarly increased composite respiration; (iii) a significantly increased lactic acid production with the higher insulin dose used.

In the case of the insulin deprived group insulin treatment *in vitro* resulted in an increased glucose uptake, lactic acid production and composite respiration (Table 2). The results from the first series of experiments suggested that tissue slices from the insulin deprived rats were more sensitive to insulin than those from normal lactating tissue. Thus a further series of comparison experiments was carried out with a range of doses of insulin designed to clarify this point. Tables 4 and 5 show the results obtained, indicating that the tissue from the insulin deprived rats is more sensitive to insulin *in vitro* than that from normal lactating rats.

The tissues from the insulin replacement group showed responses to *in vitro* insulin which were similar to those shown with normal lactating tissue, except that higher dose of insulin used did not give rise to an increased lactic acid production.

TABLE 4.

Comparison of responses to insulin by mammary slices from normal and alloxan diabetic rats. Composite respiration.

		Normal lactating				Alloxan diabetic				
Rat No.	Blood glucose mg. %	Control	Insulin μg./ml. 0·125	0·25	0·5	Blood glucose mg. %	Control	Insulin μg./ml. 0·125	0·25	0·5
1	110	249+	252	272	322	660	108	153	158	192
2	110	360	314	335	382	660	61	92	113	125
3	105	180	178	177	175	425	80	91	92	108
4	110	196	183	240	253	585	106	147	143	176
5	100	183	196	198	250	540	82	121	129	138
Means		234	225	244	276		87	121	127	148
Variance ratio*		F=6·83; P<·01>·001				F=29·26; P<·001				
"t" tests		Insulin μg./ml. "t" P 0·125 v. control 0·74>·4 0·25 v. control 0·82>·4 0·5 v. control 3·44<·01>·001				Insulin μg./ml. "t" P 0·125 v. control 5·23<·001 0·25 v. control 6·15<·001 0·5 v. control 9·38<·001				

[1] μl./100 mg. moist weight tissue/120 minutes.

+ Each value is the mean of the two observations.

* Variance ratio (F) = $\frac{\text{mean square between treatments}}{\text{Interaction mean square (Rats} \times \text{treatments)}}$

TABLE 5.

Comparison of response to insulin by mammary tissue from normal and alloxan diabetic rats. Glucose uptake[1]

		Normal lactating				Alloxan diabetic				
		Control	Insulin μg./ml.				Control	Insulin μg./ml.		
Rat No.	Blood glucose mg. %		0·125	0·25	0·5	Blood glucose mg. %		0·125	0·25	0·5
1	110	2155+	2105	2375	2860	660	780	1165	1185	1604
2	110	2645	2360	2460	2920	660	720	875	1095	1405
3	105	1275	1250	1320	1150	425	815	885	890	1070
4	110	1805	1585	1980	2215	585	700	920	940	1400
5	100	1530	1605	1560	1810	540	510	789	760	960
Means		1882	1781	1939	2191		705	925	974	1295
Variance ratio*		F=5·09; P<·05>·01				F=24·55; P<·001				
"t" tests		Insulin μg./ml. "t" P 0·125 v. control ·92>·3 0·25 v. control ·52>·6 0·5 v. control 2·83<·02>·01				Insulin μg./ml. "t" P 0·125 v. control 3·16<·01>·001 0·25 v. control 3·87 <·01>·001 0·5 v. control 8·49<·001				

[1] μg. uptake/100 mg. moist weight tissue/120 minutes.

+ Each value is mean of two observations.

* Variance ratio (F) = $\frac{\text{mean square between treatments.}}{\text{Interaction mean square (Rats} \times \text{treatments)}}$

DISCUSSION.

The effects of induction of a diabetic state clearly resulted in restriction of lactational performance similar in many ways to that resulting after hypophysectomy, e.g. decrease in weight of, and increase in mortality rate in, litters (see Folley, 1956). It is unlikely that the effects observed are due to a direct effect of alloxan on the mammary gland tissue since (a) the precaution was taken of giving the alloxan some days before the activity of the glands was tested, and (b) insulin replacement resulted in some appreciable improvement in lactational performance.

Apart from the overall index of mammary gland activity measured by litter weight progress, the measurements of metabolic activity of mammary gland slices also indicate that similar effects are found after alloxan treatment as after removal of the pituitary. Thus mammary gland slices from insulin deprived rats, on incubation *in vitro*, exhibit a decreased glucose uptake and an increased lactic acid production compared with slices from normal lactating rats. These effects are qualitatively the same as are observed after hypophysectomy although quantitatively less (Bradley, 1958).

A different effect is observed, however, with respect to the ability of the tissue to respond to insulin *in vitro*. After hypophysectomy the response of mammary gland slices to insulin *in vitro* is reduced within 48 hours to virtually nil. After alloxan, however, the tissue shows an increased sensitivity to insulin *in vitro*, a result similar to that observed with another insulin-sensitive tissue, diaphragm muscle (Krahl and Cori, 1947; Villee and Hastings, 1949). Thus, although the biochemical lesions induced by hypophysectomy and alloxan-diabetes are similar they are not identical.

Removal of the pituitary *in the rat* apparently results in lower plasma insulin activity. That such a decreased blood concentration of insulin is not solely responsible for the alterations observed in the metabolic activity of lactating mammary gland after hypophysectomy is indicated by the different responses obtained to insulin during *in vitro* incubation of slices from alloxan-diabetic or hypophysectomized rats. Thus it seems likely that some pituitary factor is necessary for the expression of the *in vitro* effect of insulin on mammary gland slices.

The principal effect observed with insulin replacement is that the mammary gland tissue produces only small amounts of lactic acid, comparable with that from normal lactating tissue, indicating repair of a biochemical lesion probably involving tricarboxylic acid cycle function. For instance, the amount of lactic acid formed in the case of normal tissue corresponds to approximately 7 p.c. of the glucose used, in alloxan-diabetic rats 60 p.c. and in the insulin replacement group only 10 p.c. Also, the fact that the amounts of lactic acid liberated in normal and insulin replacement tissue cannot account for the positive composite respiration observed, whereas in the insulin deprived group they do, indicates that insulin replacement mammary tissue tends towards the high respiratory quotient values typical of normal actively synthesizing slices of lactating mammary gland.

However, similar results in the insulin replacement group were not obtained with respect to glucose uptake. This result may be due to the inconstant nature of the insulin treatment as compared with normal secretion. Thus the results would be explicable were it found that the mechanism of uptake of glucose by the mammary gland cells is very much more sensitive to fluctuating blood insulin levels than the mechanism controlling lactic acid accumulation in this tissue.

Summarizing, the principal defects of mammary gland metabolism following hypophysectomy which have been demonstrated to date are: (1) increased production of lactic acid; (2) decreased respiratory quotient; (3) decrease in glucose uptake; (4) decrease in sensitivity to insulin *in vitro*.

The results of the present investigation indicate that, in rats, the first and second of these effects could be due to decreased insulin activity following hypophysectomy. It is doubtful whether the third is such a simple phenomenon since

insulin replacement therapy did not regularly restore glucose uptake to near normal values and the effects of induction of an alloxan-diabetic state are not so marked as those following hypophysectomy. With respect to the fourth point there is no doubt that decreased plasma insulin activity following hypophysectomy could not alone produce a decreased sensitivity to insulin *in vitro* and that some other factor, most likely the deprivation of a pituitary factor, is involved.

Replacement of pituitary hormones has not as yet proven satisfactory in maintenance of lactation after hypophysectomy of lactating rats (Cowie, 1957). Thus the possibility should be considered that insulin may be one of a group of substances necessary for complete replacement in such cases. Randle (1955) has shown that replacement with growth hormone immediately after hypophysectomy leads to near normal plasma insulin activities. If hypophysectomy results in a simple inadequate plasma insulin activity, restored by growth hormone, correction of the metabolic lesion at the lactic acid level would be expected. However, such growth hormone replacement therapy after hypophysectomy of lactating rats results in a slightly increased glucose uptake by mammary gland slices with no effect on the lactic acid production (Bradley, 1955). Thus it is suggested that an interaction between a pituitary factor and insulin is involved.

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THE RELEASE OF HISTAMINE FROM THE MAST CELLS OF THE RAT

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SUMMARY.

A method is described for the isolation of mast cells from the peritoneal cavity of the rat. Histamine was released from the mast cells by a number of chemical substances, mechanical trauma, osmotic pressure and extracts prepared from nucleated tissues. The chemical nature of the active principle in splenic tissue extract appeared to be an arginine-containing polypeptide.

INTRODUCTION.

The mast cells are an important source of histamine and in most tissues the histamine content can be related to the number of mast cells present (Riley and West, 1953). Tissues may be depleted of histamine by repeated injections of compound 48/80, a substance known to release histamine from tissues (Riley and West, 1955). Coincident with the fall of tissue histamine, the mast cells disappear from the tissues. In the recovery phase the tissue histamine level increases and mast cells reappear.

Riley (1953) demonstrated histological changes in the mast cells varying from cellular swelling to complete disintegration following the intravenous injection of histamine release agents. Some of the agents used were fluorescent and the mast cells became fluorescent prior to disintegration. Riley concluded that these agents entered the mast cells and caused the cells to disrupt and release histamine.

The object of the work which is reported in this paper was to investigate *in vitro* the phenomenon of histamine release from mast cells. A preliminary report of this work has been published elsewhere (Archer, 1958).

MATERIALS AND METHODS.

Mast cells were obtained from the peritoneal cavity of adult Wistar strain albino rats. Under ether anaesthesia and with the animal on its back, 40 ml. sterile physiological saline were injected into the peritoneal cavity in the midline above the symphysis pubis. After rocking to distribute the saline the rat was placed on a sloping board to drain the solution

into the lower abdomen. A gauge 14 S.W.G. needle was inserted into the lower part of the abdominal cavity and the fluid collected into a centrifuge tube. Blocking of the needle by the mesentery was less likely if a number of holes were cut in the shaft of the needle near its point. The tubes were centrifuged, the supernatant fluid discarded, and the button of cells dispersed in two or three drops of physiological saline solution.

These preparations from the peritoneal cavity contained mast cells, leucocytes, endothelial cells and a number of red cells varying with the amount of trauma to the abdominal wall. Approximately 0·5 million mast cells were obtained from each rat.

A small amount (approximately 0·25 ml.) of the cell suspension was placed on the top of a column of 50 p.c. human albumin in a Wintrobe haematocrit tube and the tube centrifuged. The albumin solution was prepared as described earlier (Archer and Kooptzoff, 1958). The mast cells passed through the albumin solution to the bottom of the tube and the other cells remained on the top. The separated mast cells are shown in Fig. 1.

Tissue extracts were prepared by grinding the tissue with an equal volume of physiological saline solution in a cylinder and rotating piston type of homogeniser. The homogenate was incubated at 37° C. for 30 minutes and cell debris was removed by centrifugation.

Compound 48/80, a powerful synthetic histamine release substance, is a condensation product of p-methoxyphenethylmethylamine and formaldehyde. It was obtained from Messrs. Burroughs Wellcome & Co. (U.S.A.) Inc. The powder was dissolved in physiological saline solution.

Protamine sulphate solution (1 *p.c.*) was obtained from Evans Medical Supplies Ltd. and was diluted with physiological saline to give a solution containing 10 μg/ml.

Peptone (*Bacteriological Peptone, P.D. & Co.*) was dissolved in physiological saline solution to a concentration of 10 mg. peptone per ml. saline solution.

d-Tubocurarine (*"Tubarine"*) *solution* was prepared to contain 1 mg. d-tubocurarine per ml. of saline solution.

Trypsin 1:250 (*Difco*), *Papain* (*Difco*) *and Pepsin* 1:2500 *B.P.* (*P.D. & Co.*) were dissolved in physiological saline solution to a concentration of 5 mg./ml. Cystine was added to the papain solution.

Histone was prepared from rat spleen by the method of Crampton, Lipshitz and Chargass (1954).

Histamine measurements were performed by the method of Lowry, Graham, Harris, Priebat, Marks and Bregman (1954). Briefly, the method is to add trichloracetic acid to the material being assayed and to adsorb histamine on a Decalso column. The histamine is eluted from the column with hot potassium bromide. 2:4-Dinitrofluorobenzene is then added to give a yellow colour with histamine and other amines. The histamine product is then extracted with methyln-hexyl ketone and finally extracted with hydrochloric acid. The density of the yellow colour is measured colorimetrically.

RESULTS.

Histamine content of mast cells. Separated mast cells were assayed for histamine by the method of Lowry *et al.* (1954). The cells contained approximately 36 micrograms per million cells.

Liberation of histamine by maceration of mast cells. Approximately one million mast cells in saline were macerated mechanically in the cylinder and rotating piston type of homogeniser until few intact cells remained. Histamine was not detected in the supernatant of the intact mast cells, but was present in the supernatant after maceration of the cells. A small amount of histamine remained in the deposit.

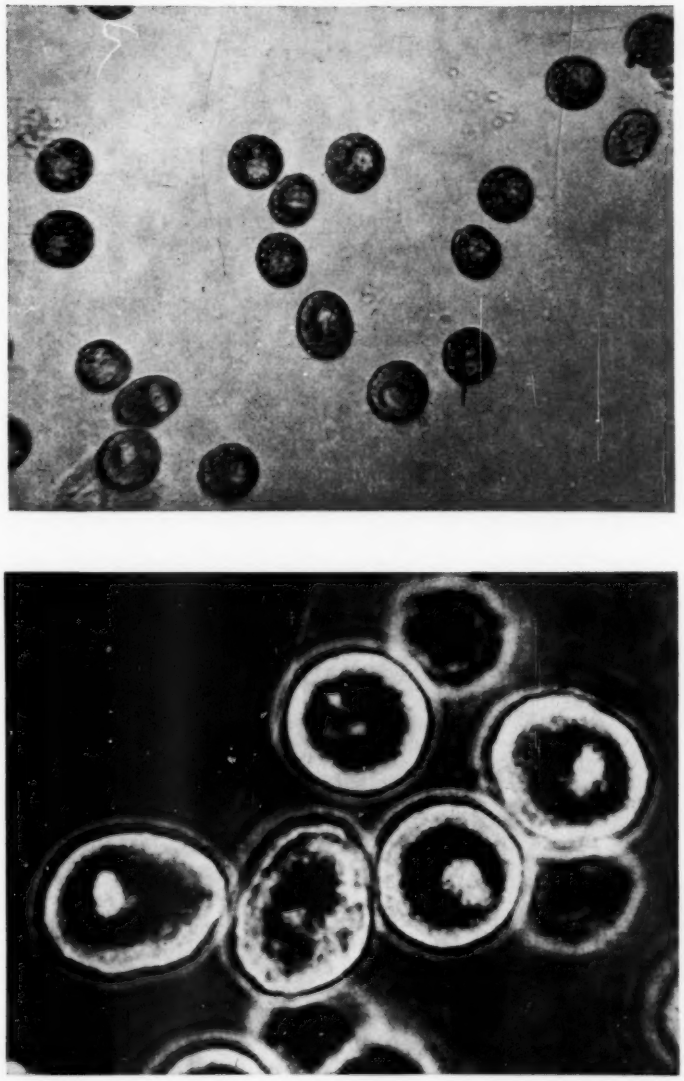

Fig. 1. Normal mast cells from the peritoneal cavity of the rat in physiological saline solution.

(a) White light illumination, unstained, X-300 (approx.).

(b) Phase contrast illumination, unstained, X-1000 (approx.).

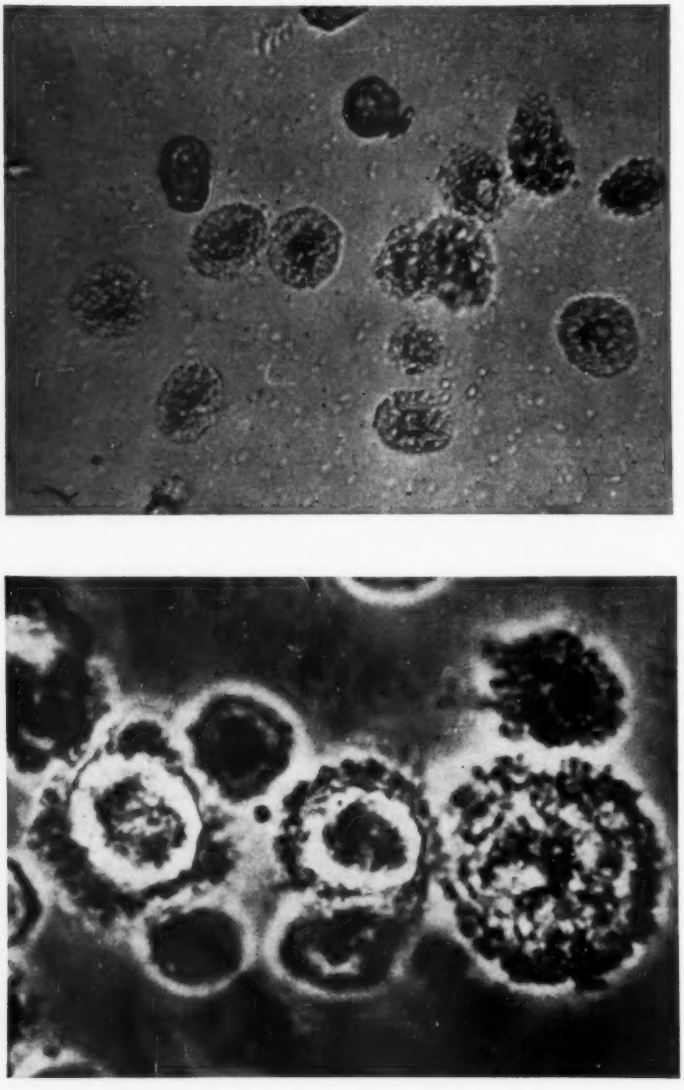

Fig. 2. Disrupted mast cells in physiological saline solution containing 5 μg/ml. protamine sulphate.

(a) White light illumination, unstained, X-300 (approx.).

(b) Phase contrast illumination, unstained, X-1000 (approx.).

Liberation of histamine with distilled water. The addition of distilled water caused the mast cells to disrupt and histamine was detected in the supernatant solution. A small amount of histamine was also found in the disrupted cells.

Effect of compound 48/80 on mast cells. Immediately after the addition of an equal volume of compound 48/80 (20 μg/ml.) many of the mast cells showed gross changes, similar to the changes seen in Fig. 2. The cell membrane was less distinct and in many instances the granules appeared to have escaped, forming a halo round the disrupted cell. The results of histamine estimations performed in two experiments are shown in the Table. The mast cells used in these experiments had been collected from rats approximately 20 minutes before the addition of compound 48/80.

TABLE 1.

The release of histamine from mast cells by compound 48/80.

	Experiment I	Experiment II
Number of mast cells in approximately 0·5 ml. saline	720,000	1,000,000
Concentration of compound 48/80 in mixture	10 μg./ml.	10 μg./ml.
Histamine in supernatant after addition of compound 48/80	13·8 μg.	24·0 μg.
Histamine remaining in deposit	12·7 μg.	17·0 μg.
Percentage histamine released	52	58

It was found that mast cells in saline rapidly became refractory to compound 48/80 and other histamine release agents. Only a small percentage of the cells disrupted and only trace amounts of histamine were released when the cells had been collected from the rat more than 45 minutes prior to the addition of the release agent.

Disruption of mast cells with the release of histamine was also caused by protamine sulphate, d-tubocurarine, trypsin with cystine, peptone and rat spleen histone. Papain and pepsin were found to cause disruption of mast cells, but histamine assays were not performed in experiments with these enzymes.

Effect of rat splenic tissue extract on mast cells. Microscopic examination of the mast cell suspensions after the addition of an equal volume of splenic tissue extract revealed histological changes in the mast cells similar to those described following the addition of compound 48/80. Histamine was detected in the supernatant solution but was not found in the tissue extract prior to the addition of mast cells. Similar changes in mast cells followed the addition of extracts of the following tissues: rat kidney, liver, lung and heart; mouse spleen, kidney, liver and lung; rabbit spleen, kidney, liver and lung; pigeon red cells and human white cells. Histamine was present in the supernatants after the addition of mast cells to pigeon red cell and human white cell extracts, but not

in the supernatants after the addition of mast cells to human and rat red cell extracts.

Nature of the active principle in the splenic tissue extract. The active principle in the splenic extract was not destroyed by boiling and was stable in acid solution (0·25 N perchloric acid for 15 minutes at 90° C.) and in alkaline solution (0·3 N potassium hydroxide for 24 hours at 20° C.).

Partial sedimentation of the active principle occurred on ultra-centrifugation of the extract at 100,000 *g* for two hours. Dialysis against distilled water resulted in a gradual loss of activity.

The splenic tissue extract was boiled and treated with perchloric acid and then fractionated with 25 p.c. ethanol at − 5° C. A precipitate formed and was discarded. The supernatant fluid was dialysed against large volumes of distilled water for 12 hours and dried in the frozen state. The powder was dissolved in a small amount of physiological saline solution and contained the active principle in high concentration. It gave a negative reaction for carbohydrate (Molisch's test), a positive biuret reaction for protein and a positive Sakaguchi reaction for arginine. No nucleic acid was detected by U.V. absorption. The antiheparin activity of the concentrated solution was assayed by the antithrombin method of Monkhouse and Jacques (1950) and the solution was found to have a neutralizing effect equivalent to approximately 20 international units of heparin per ml.

Action of histones on mast cells. The above results suggested that the active principle might be related to histone. For this reason histone was prepared from rat spleen and was found to disrupt mast cells in a manner similar to the tissue extracts. Solutions of splenic histone and the active principle from splenic tissue were diluted to the same antiheparin titre. It was found that the two solutions had equal activity against mast cells as measured by the highest dilution at which disruption of added cells occurred.

DISCUSSION.

The results support the thesis propounded by Riley (1953) that histamine release agents cause mast cell disruption with the release of histamine from the disrupted cells. Mast cell disruption caused by mechanical trauma and osmotic pressure also was shown to release histamine from these cells. It seems that histamine is loosely bound in the mast cells and disruption of the cell by a number of agents releases most of the histamine.

A substance was found in extracts prepared from macerated nucleated tissues of the rat, mouse, rabbit, human and pigeon, which caused disruption of added mast cells. In the case of rat splenic extract, human white cell extract and pigeon red cell extract, it was shown that mast cell disruption was associated with the release of histamine into the surrounding solution.

The factor isolated from tissues is of interest because it may represent the trigger in tissue injury for the release of histamine. A working hypothesis is as follows:

Injury to tissue
↓
Release of factor from damaged nucleated cells
↓
Action of this factor on mast cells
↓
Release of histamine

The results reported in this paper suggest that the active factor is released only from nucleated cells and that it may be released by traumatic or other damage. The chemical nature of the factor has not been clearly determined but the evidence suggests that it is an arginine-containing polypeptide. It is possibly derived from histone.

There are two theories currently held concerning the release of histamine in the body. The first is that histamine is released as a result of cellular injury and the evidence presented in this paper tends to support and extend this theory. The second theory is that histamine is released from tissues by a factor produced in plasma following the addition of various substances such as immune precipitins, agar, starch, inulin, zymosan and dextran. No mention has been made in the present paper of investigations into the plasma theory, but it has been found that rat plasma to which dextran has been added causes disruption of mast cells and release of histamine. It is therefore possible that both release mechanisms may operate through the mast cell and this is being further investigated.

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MEASUREMENT OF ANTIPYRINE IN PLASMA: INTERFERENCE BY CERTAIN DRUGS

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SUMMARY.

It has been shown that certain drugs cause interference in the antipyrine reaction. Such drugs are chloramphenicol, tetracycline, sulphonamides, chlorpromazine, pentobarbitone and possibly structural analogues containing a benzamine group.

Antipyrine space should not be measured in the presence of such drugs, especially in anuric patients where drugs are retained and may accumulate. During anuria, injected creatinine is preferable for measuring total body water.

A complete pre-injection plasma blank is invariably raised when these drugs are present and should be included in each estimation of antipyrine space.

Sulphuric-acid-filtrate blanks may be useful in indicating any change in the level of these drugs in the plasma after antipyrine has been injected. These blanks may fortuitously correct for the interference caused by conjugated chloramphenicol, but not necessarily for other drugs.

INTRODUCTION.

Antipyrine space is now widely used to measure total body water (Soberman *et al.*, 1949; Prentice *et al.*, 1952; Farber and Soberman, 1956; Petersen, 1957). The material is injected, four blood samples collected over 12-24 hours, and the rate of fall of plasma antipyrine level observed. From this information the volume of distribution is readily calculated.

Antipyrine is usually measured in plasma by the original method of Brodie *et al.* (1949). In this method sulphuric acid and sodium nitrite are added serially to protein-free plasma filtrates, and the resulting 4-nitroso-antipyrine is determined absorptiometrically at 350 mμ. The optical densities of all solutions are read on two occasions; firstly, after the addition of sulphuric acid and finally when the reaction is complete 20 minutes after adding nitrite. The spectrophotometer is set at zero in the first series of readings with water and sulphuric acid as reference, and in the second series with water and sulphuric

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acid and nitrite. There is an appreciable difference in the optical densities of these two reference solutions. The sulphuric-acid-filtrate (s-a-f) blank is subtracted from the final reading to obtain the amount of antipyrine in the solution. The method was stated to be specific for antipyrine and to give negligible values for normal plasma obtained before injection of antipyrine.

Difficulties have been encountered with this method. Some workers claimed that it was necessary to take repeated readings of optical densities since variations were noted in the time taken for solutions to reach maximum extinction (Ikkos *et al.*, 1954; Kraybill *et al.*, 1954). Temperature and excess nitrite were stated to cause these variations (Davidsson and MacIntyre, 1956; Zak, 1957). Also, the use of s-a-f blanks in calculating antipyrine concentrations seemed questionable because they did not include all reagents taking part in the formation of the compound being measured.

Modifications have been introduced to overcome such difficulties, and results obtained by the modified method are given in this paper. The essential change concerned the use of a different blank. Plasma was collected prior to the injection of antipyrine, a blank was obtained by adding sulphuric acid and nitrite to the plasma filtrate, and the absorption was read against a zero setting with water and sulphuric acid and nitrite. This single, complete plasma blank replaced the multiple s-a-f blanks of Brodie *et al.* (1949), and was subtracted from all values following injection of antipyrine.

With anuric patients it was found that the modified method sometimes gave falsely high estimates of antipyrine space, and levels of antipyrinoid substances in the plasma blanks were always raised (Edwards, 1959). In a series of patients without renal disease, high plasma blank levels were also encountered on two occasions, and it was noted that these two patients were receiving sulphonamides. Interference of this nature could also have caused the aberrant results with anuric patients since drugs were administered in many of these cases and would tend to accumulate in the body fluids. Further investigation in fact revealed that erroneous results during anuria were associated with the administration of chloramphenicol or chlorpromazine. A small group of drugs was tested *in vitro*, and chloramphenicol, tetracycline, Gantrisin, Trisulpha, chlorpromazine and pentobarbitone were found to cause absorption at 345 mμ. (Edwards, 1959).

It was therefore decided to investigate three of these drugs with respect to their effect on the estimation of antipyrine. Results of these studies are reported in this paper.

Methods.

Present antipyrine method. Protein-free neutral filtrate, dilution 1 in 5, was obtained from 5 ml. of plasma, using Somogyi's zinc hydroxide method. No. 42 Whatman filter papers were found necessary to ensure a water-clear filtrate. 4 ml. of filtrate were pipetted into duplicate 20 ml. test tubes and placed in a water bath at 25° C. Filtrate from a pre-

injection sample of plasma was treated similarly, as also were water and standard solutions of 5, 10 and 15 mg. of antipyrine per litre. Nitrous acid reagent was freshly prepared by adding 1 volume of 6N sulphuric acid to 20 volumes of 0·015 p.c. sodium nitrite solution, and 1 ml. of this reagent pipetted into each test tube. After 40 minutes, the optical density of each solution (complete reaction) was read against the reagent blank at the point of maximum absorption (345 mμ), using a spectrophotometer (Hilger "Unispec", Beckman Model DU, or Bausch and Lomb "Spectronic 20"). The total plasma antipyrine content was calculated for each filtrate, and then the true plasma antipyrine level obtained by subtracting the pre-injection plasma blank from each.

The effect of drugs and anuria on the measurement of antipyrine space. An 81-year-old patient had been anuric for six days following a transurethral resection. Antipyrine space and creatinine space were estimated as described elsewhere (Edwards, 1959). During the test the patient also received intramuscular injections of chloramphenicol, 500 mg. every six hours, and had been given eight doses previously. Antipyrine space was measured by the present method and also by Brodie's method and the results compared.

The effect of drugs on plasma blanks and recoveries. Plasma samples from 34 patients with normal renal function and from 16 patients with anuria were tested for blank values and recovery of antipyrine. In addition, plasma was tested from three patients who were receiving regular doses of one of three drugs. One of these patients was anuric, and was receiving injections of chloramphenicol, 500 mg., every eight hours. The other two were not anuric; one was receiving Trisulpha (1·0 gm. every six hours) and the other chlorpromazine (50 mg. every nine hours).

RESULTS.

Plasma blanks and recoveries. Normal values for plasma blanks and recoveries with the present method are shown in Table 1. Recovery of antipyrine added to the plasma of normal or anuric patients was satisfactory. Plasma from anuric patients gave a high blank reading, ranging from normal to 30 mg./l.

TABLE 1.

Modified antipyrine method: plasma blanks in normal and anuric patients and recovery of added antipyrine.

Subjects	Mean ± S.D. (no. of patients)	
	Plasma blank (mg./l.)	Recovery of antipyrine added to plasma blank (per cent)
Normal	2·8±1·2 (34)	96·1±1·7 (17)
Anuric	14·1±5·5 (16)	96·5±1·5 (12)

The error of the method was calculated as an optical density reading of 0·001, which was 0·4 p.c. of the mean optical density of 0·234 for 30 pairs of duplicates. Duplicates were taken of the filtrates, after plasma proteins had been precipitated.

The effect of drugs and anuria on the measurement of antipyrine space. Details of an experiment in an anuric patient who was receiving chloramphenicol

are given in Tables 2, 3, and 4. Table 2 shows the rise in the plasma blank with time (column 5), and the apparently fortuitous correction of most of this rise by the s-a-f blank using Brodie's method. It can be seen that the s-a-f blank continued to rise, and changed from an optical density of ·015 to ·088 in six

TABLE 2.

Administration of chloramphenicol (500 mg. 6-hourly) during anuria: effect on sulphuric-acid-filtrate blanks and on plasma blanks.

Time before or after injection of antipyrine (hours)	Optical density at 345mμ		Plasma blank expressed as mg. of antipyrine	
	s-a-f blank*	complete reaction*	Brodie's method*	Present method*
−67	·015	·023	1·4	4·0
−19	·040	·051	1·9	8·9
−1	·053	·063	1·8	11·0
+4	·055	**	**	**
+8	·055	**	**	**
+23	·069	**	**	**
+71	·088	**	**	**

* See section on Methods in this article for explanation of these terms.
** Blanks not able to be measured following injection of antipyrine.

TABLE 3.

Administration of chloramphenicol during anuria: effect on measurement of levels of antipyrine in plasma.

Time after injection of antipyrine (hours)	Total plasma antipyrine, including plasma blank (mg./l.)		True plasma antipyrine† (mg./l.)	
	Brodie's method*	Present method	Brodie's method	Present method
Before	1·8	11·0	—	—
4·1	40·6	49·7	38·8	38·7
8·1	36·9	46·0	35·1	35·0
23	25·4	37·1	23·6	26·1
71	9·8	25·1	8·0	14·1

* Complete reaction minus s-a-f blank; for corresponding s-a-f blanks, see Table 2.
† Total plasma antipyrine minus pre-injection plasma blank; the latter is assumed to remain constant during the experiment.

TABLE 4.

Administration of chloramphenicol during anuria: effect on estimation of total body water by antipyrine.

Method	Antipyrine space (l.)		
	4-8 hr.	8-23 hr.	23-71 hr.
Present method	36·4 (107 p.c.)	37·3 (110 p.c.)	44·0 (130 p.c.)
Brodie's method	36·3 (107 p.c.)	35·2 (104 p.c.)	39·1 (115 p.c.)

Figures in brackets express the antipyrine space as a percentage of the total body water. T.B.W. was measured by the creatinine method, which was found to be more reliable during anuria.

days. The effect of this on the measurement of plasma antipyrine levels can be seen in Table 3. With Brodie's method, where the s-a-f blanks rose steadily, a changing correction resulted for each estimation of plasma antipyrine level. With the present method, a pre-injection plasma blank was measured, and this value subtracted from total plasma antipyrine levels obtained after injection; the plasma blank was assumed to remain constant, as it could not be measured

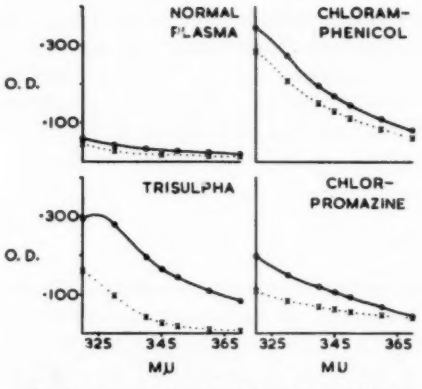

Fig. 1. Curves of optical densities of filtrates of normal plasma and of plasma from patients receiving chloramphenicol, "Trisulpha", or chlorpromazine, read at 320-370 mμ.
Closed circles—"plasma blank", complete reation; optical densities read 40 minutes after addition of nitrous acid reagent.
Crosses—"sulphuric-acid-filtrate blank"; optical densities read 40 minutes after addition of sulphuric acid.

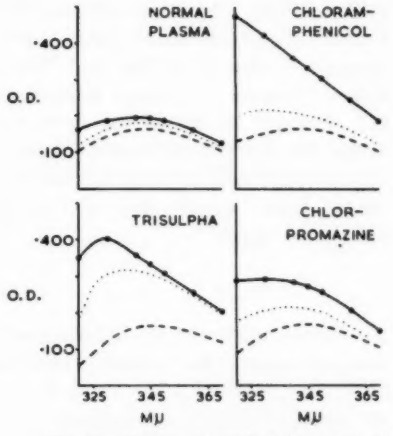

Fig. 2. Curves of optical densities of filtrates of the same plasma samples as shown in Fig. 1, after addition of 30 mg. of antipyrine per litre to each sample during protein precipitation.
Closed circles—complete reaction (after addition of nitrous acid reagent).
Broken line—resultant after subtraction of plasma blank values (Fig. 1) from full reactions.
Dotted line—resultant after subtraction of sulphuric-acid-filtrate blanks from full reaction.

again once antipyrine had been injected. Thus the values for plasma antipyrine measured by these two methods differed, and the difference increased with time (Table 3). The measurements of total body water by antipyrine in the presence of changing levels of chloramphenicol are shown in Table 4. Antipyrine space is compared with a separate estimate of total body water of 33·9 litres, using the creatinine method previously described (Edwards, 1959).

Plasma blanks and recoveries on plasma from patients receiving drugs. In three patients receiving drugs (chloramphenicol, Trisulpha, or chlorpromazine), the plasma blanks by either method and also the s-a-f blanks were all greater than in normal plasma (Fig. 1). With normal plasma the s-a-f blank (dotted line) was close to the complete reaction (plasma blank of the present method). In the presence of drugs there was a variable difference between the two blanks. With Trisulpha and chlorpromazine the s-a-f blank gave little indication of the interference caused by these drugs. With conjugated chloramphenicol, however, the relationship was close enough to enable the s-a-f blank to correct for some 90 p.c. of the interference. The shape of the absorption curve over a range of wavelengths can be seen to differ in each case, and this is accentuated by the curves obtained in Fig. 2, where 30 mg. of antipyrine/l. had been added to each of the four plasma samples. If the corresponding plasma blanks (complete reaction, Fig. 1) were subtracted from the recovery curves (complete reaction, Fig. 2), almost identical curves were obtained in each case for true plasma antipyrine (broken lines, Fig. 2). The latter curves were similar to the curve obtained for pure antipyrine in water, with maximum absorption at 345 mμ. Thus the recovery of antipyrine from these four plasma samples was satisfactory when the respective plasma blank levels were known. The recovery curves obtained by subtracting s-a-f blanks (Brodie's method) are shown in Fig. 2 by dotted lines. In the case of Trisulpha, there was no resemblance to the true antipyrine curve.

DISCUSSION.

There seems to be little doubt that certain drugs can interfere with the antipyrine reaction. Firstly, erroneous estimates of total body water by the antipyrine method were obtained in anuric patients who had chloramphenicol or chlorpromazine administered during the test. If antipyrine is used in patients who are ill or abnormally hydrated, it has been found necessary to collect blood samples for 12-24 hours after injection (Farber and Soberman, 1956). This period allows sufficient time for several doses of such drugs to be given. Remenchik *et al.* (1958) have measured the antipyrine space in anuric patients, using Brodie's method. They reported values greater than 60 p.c. of the body weight in five out of eight patients, but made no comment about the antipyrine method. Secondly, high plasma blanks noted with the present method were found consistently in anuric patients (Table 1), and occasionally in normal

subjects. Their occurrence was clearly related to the administration of one or more of the drugs listed in the Introduction. Thirdly, raised s-a-f blanks have been previously recorded by Reid *et al.* (1958), but only for intestinal contents of rabbits. Raised s-a-f blanks have been seen with the three drugs tested here (Fig. 1), and may give some indication of alterations in the level of such a drug in the plasma during measurement of the antipyrine space (Table 2). This would be useful after the injection of antipyrine, when plasma blanks can be no longer obtained.

Thus the main error is caused by a change in level of these drugs in the plasma after the blood sample for the pre-injection plasma blanks has been taken. It would seem that Brodie's s-a-f blanks correct for most of the interference caused by conjugated chloramphenicol (Table 4), but not for other drugs (Fig. 2).

If such drugs must be given, they should, if possible, be suspended some days before antipyrine is injected. In anuric patients repeated plasma blanks before the test should then show an unchanging level, and in non-anuric patients the plasma blank should have fallen to normal. In all patients in whom antipyrine space is to be measured, a pre-injection plasma blank should be included. If the plasma blank is found to be raised above the normal range (Table 1), interference in measurement of antipyrine space can be expected. Finally, s-a-f blanks should be used to indicate whether the plasma blank remains constant during the test, but the validity of including this incomplete blank in calculations of the true antipyrine content of plasma is doubtful.

Acknowledgment. It is a pleasure to acknowledge the help given by Dr. B. D. Stacy, Assistant Biochemist, Sydney Hospital, in discussion and preparation of this paper.

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THE DYNAMIC RED CELL STORAGE FUNCTION OF THE SPLEEN IN SHEEP

I. RELATIONSHIP TO FLUCTUATIONS OF JUGULAR HAEMATOCRIT

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SUMMARY.

Fluctuations and anomalous changes of jugular haematocrit were commonly observed in sheep. Experimentally, the jugular haematocrit of sheep responded to soothing, to isolation from visual and auditory stimuli, or to Pentobarbitonum anaesthesia by decrease, and to excitement, to exercise, or to intravenous adrenaline or *nor*-adrenaline by increase. By observing the effect of splenectomy upon haematocrit responses, and by relating responses in intact sheep with changes in red cell numbers in the spleen, as deduced by external gamma radiation counting after preparative infusions of ^{51}Cr-labelled red cells, it was concluded that these effects were due to inverse changes in the degree of filling or contraction of the spleen. By relating the weight of the ablated spleen with jugular haematocrit before operation and at the moment of pedicle ligation it was calculated that, in the conscious sheep in an unalarming environment, the spleen may contain up to one-seventh of the total blood volume and one-fourth of the total red cell volume. Analysis of the appearance of ^{51}Cr-labelled red cells in the splenic area after infusion into the jugular vein indicated that red cells accumulate in large numbers in the spleen, that individual cells are retained there for periods ranging up to 30 minutes or even longer, and that temporarily retained cells are subsequently displaced and replaced by cells from the afferent blood. A state of dynamic red cell storage therefore exists. The plasma separated from the concentrated red cells rejoins the blood mainly within the spleen. Perfusion experiments with the isolated spleen are consistent with control of intrasplenic red cell concentration by vascular shunts rather than by distal occluding sphincters. The formal difficulty of defining a normal value for jugular haematocrit of individual sheep, and the necessity for training, quietness and gentleness when handling sheep, are stressed.

INTRODUCTION.

During investigations in 1955 into the toxic effects of excessive consumption of wheat grain by sheep, fluctuations and anomalous increases in haematocrit values of successive samples of jugular blood were frequently observed. First, among sheep which had been recently removed from their companions for use in experiments, haematocrits of samples collected after about an hour were often considerably less than in samples collected immediately after isolation.

Similarly, when successive samples were collected during determination of blood volume, haematocrits typically became progressively less, so that in the 20-minute samples they might be 10 p.c. less than in the initial samples. These effects could not be explained in terms of water transfer from the blood, since they were not accompanied by commensurate changes in plasma specific gravity. Secondly, after heavy wheat consumption haemoconcentration increased in intact sheep during the first 12 hr. at a rate and to an extent greater than could be accounted for simply as a consequence of the progressive decrease of plasma volume, whereas this was an adequate explanation in splenectomized sheep. Recalling the classical studies of Barcroft and his school in the period 1925-30 (see Knisely *et al.*, 1948) upon the red cell storage function of the spleen in dogs and cats, we assumed that a similar mechanism operated in sheep, and that this explained the phenomena observed. However, a search of the literature failed to show that red cell storage function of the spleen had been formally demonstrated in sheep.

Furthermore, it appeared that Barcroft's conclusions in respect to the spleen of dogs had been weakened by the subsequent demonstration by Hahn *et al.* (1942) of so-called "rapid mixing" in the spleen. It was therefore decided to examine the relationship of the spleen to haematocrit changes in sheep.

Thomson *et al.* (1946), unknown to us at the time, had observed fluctuations of jugular haemoglobin values, and had associated them with various degrees of excitement and exercise. However, they had specifically rejected the possible rôle of the spleen because subcutaneous injection of adrenaline had not been followed by increased jugular haemoglobin concentration, and because they considered the spleen in sheep was too small to cause effects commensurate with those observed; and they stated that "a decrease in plasma volume shown to occur in man after exercise . . . seems more likely".

Since the relatively inaccessible site of the spleen in the sheep made it difficult to apply the radioscopic-delineation and exteriorization techniques devised by Barcroft, experiments were largely confined to comparing the effect of various procedures upon the haematocrit value in intact sheep and in splenectomized sheep. In the final stages, after the appearance of a paper on the spleen in goats by Anderson and Rogers (1957), the main conclusions were reinforced, and important new information was added, by external counting of gamma radiation after injection of red cells labelled with ^{51}Cr.

Materials and Methods.

The sheep were mainly Merinos brought in from a field station, and kept usually in small groups in pens. Some had been removed only a short time, and still resented handling; others had been at the laboratory for many months, and were more accustomed to the presence of attendants and to handling. Usually blood was withdrawn from a jugular vein after piercing with a hypodermic needle attached to a syringe. Dried heparin was used as anticoagulant. The samples were centrifuged for 60 min. in calibrated Wintrobe tubes,

of internal diameter 3 mm., at 5000 r.p.m. and radius of gyration 17 cm. at the bottom of the tubes, corresponding to RCF = 4756 times gravity, and readings were to the nearest one-tenth of a division. With this procedure, differences of 0·17-0·20 between the means of duplicates in the range 20-40 are significant at the 5 p.c. level, and the trapped plasma correction factor is 0·97 (Hodgetts, 1959). Haematocrit values thus corrected are expressed here for convenience as absolute numbers, but are understood as percentages. Plasma specific gravity (PSG) was estimated by the copper sulphate method of Phillips *et al.* (1945), and plasma protein (PP) concentration, in g./100 ml., was calculated from the formula, PP = 360 (PSG — 1·0070). When PSG changed during a moderately short series of observations, it was assumed that this was the result of water transfer and, when required, the contribution of this factor was eliminated by means of the following formula:

$$H' = \frac{100 \cdot H}{(100\text{-}H) \cdot \frac{PP_2}{PP_1} + H}$$

where H = haematocrit value, H′ = H adjusted for water transfer, PP_1 = initial PP, and PP_2 = subsequent PP.

Red cells were labelled with ^{51}Cr as sodium chromate by the method described by Gray and Frank (1953). Uptake was of the order of 36-60 p.c. after 1¼-2 hr. at room temperatures. Sufficient washed labelled cells were re-injected into a jugular vein to lead to satisfactory counts over the splenic region. In various experiments, from 10 to 20 ml. of packed red cells, labelled with 40-130 μC of activity, were injected within 1-3 min. For counting the gamma radiation, a scintillometer (Model DS-1; Nuclear Instrument and Chemical Corporation, Chicago, U.S.A.) was employed, fitted with a thallium-activated sodium iodide crystal ¾ in. diam. and ¾ in. long. In order to increase counting rate and thereby improve precision, the heavy directional shield was removed. The instrument was lightly but firmly held against the animal's body at a position over the splenic area which yielded maximum counts. The output was fed to an amplifier, and was read on the rate-meter. Readings were subject to fluctuations, depending upon varying pressure upon the body, movements of the animal within its rather loose skin, and other factors; but with experience and care acceptable results were obtained. Since ^{51}Cr-labelled red cells are distributed throughout the blood like normal cells and are not selectively retained in the spleen (Kraintz *et al.*, 1957, 1958), surface counts are proportional to the number of red cells, labelled or not, within range of the crystal, being influenced by the vascularity, extent, thickness, and distance of the underlying structures, and by the red cell concentration in their contained blood.

In the case of the sheep's spleen, of which the area is many times that of the scintillometer applicator, changes in surface counts may be due to changes in thickness of the organ (i.e., dilatation or contraction), to changes in mean red cell concentration in the splenic tissue subtended by the crystal, or to both.

Adrenaline (1:1000) was used as the hydrochloride (Parke, Davis & Co.) and *nor*-adrenaline (1:1000) as the bitartrate monohydrate ("Levophed", Fredk. Stearns & Co.). Pentobarbitonum Sodium (PBS), occasionally in the form of "Nembutal", Abbott Labs., was given by intravenous injection of 29 mg./kg. body weight (BW), as a solution containing 65 mg./ml.

EXPERIMENTAL.

Evidence of high red cell numbers in the spleen in situ, *as revealed by use of ^{51}Cr.*

In a conscious splenectomized sheep injected i.v. with ^{51}Cr-labelled red cells, counts quickly rose over the whole body, and within a few minutes reached

maxima which were maintained for many hours. Counts over the site of splenectomy (1500/min.) were not significantly different from over the corresponding region on the right side or over the gluteal region. The greatest counts were obtained over the heart (2500/min.). In two conscious intact sheep (895 and 904) counts over the spleen rose quickly at first, but at a decreasing rate, and slowly reached maxima after about 30 min. (Fig. 1). The final counts reached over the spleen were very much greater than elsewhere, namely, 10 times those over muscular masses such as the thigh, and 4-5 times those over the heart. Over the remainder of the body, maximum counts were reached within a few minutes, as in the splenectomized sheep. The same general pattern was observed in an intact sheep (885) which was deeply anaesthetized by means of PBS administered 25 min. before injection of labelled red cells; counts rose quickly over the splenic region, but at a somewhat slower rate, so that after 25 min., when this animal was injected i.v. with adrenaline for other purposes, counts had reached only about 75 p.c. of the potential maximum, as described later.

These findings are consistent with the general experience based upon removed spleens in most animals, that the spleen is a highly vascular organ and that the red cell concentration in it is greater than elsewhere in the body; they also make possible deductions on the rate of displacement of splenic red cells by circulating red cells, which are discussed later.

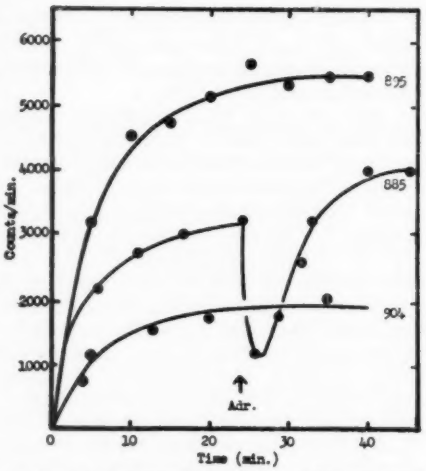

Fig. 1. Increase of gamma radiation count over the splenic region of intact conscious (895 and 904) and anaesthetized sheep (885), after i.v. ^{51}Cr-labelled red cells. At "Adr ↑", adrenaline (11 µg./kg.) was injected i.v. into the anaesthetized sheep (885).

The effect of "holding", i.e. quiet restraint, upon jugular haematocrit and external count.

When collecting blood samples for the determination of plasma volume (PV) and blood volume (BV) by the T-1824 and venous-haematocrit method, it was found convenient to hold each sheep quietly until the last sample had been collected, rather than to release and then recapture it for each withdrawal. The observations on the progressive decrease of haematocrit which usually occurred under those conditions suggested experiments, uncomplicated by dye injection and by removal of relatively large samples of blood (10-15 ml.), on the effect of quiet restraint and immobilization. For this purpose each sheep was removed

from its crate, or was seized with minimum disturbance in the small pen in which it had been kept with other sheep. A sympathetic attendant, standing in a quiet place with the animal held in the standing position between his legs, and with a hand around its neck, then gently restrained and if necessary soothed it. This procedure is here referred to as "holding". Blood samples were removed at intervals after re-puncturing a jugular vein.

In the intact sheep the typical response to "holding" was a decreasingly rapid fall of haematocrit approaching an asymptote after about 30 min. (Fig. 2). Haemodilution through water transfer, when PSG indicated that this had occurred, played only a small part in the fall. A small proportion of sheep behaved atypically, and this appeared to be related to excitable temperament; thus, No. 904 responded relatively slightly and irregularly, and No. 493 did not respond at all during 15 min. Others, not illustrated in Fig. 2, which had responded rather like No. 904 on earlier occasions, responded more typically on later occasions, probably through increasing habituation to the presence of man and to handling. Figure 2 also illustrates that the ability to respond to "holding" is not confined to sheep with high initial haematocrits; thus No. 852 responded as typically after it had been rendered relatively anaemic by a series of bleedings and plasma replacements (852b), as it had earlier (852a), when the initial value had been almost 28 p.c. greater. On the other hand, in splenectomized sheep with initial values ranging from 25 to 35, haematocrit fell very little or not at all after "holding", and usually (e.g. No. 507) the slight decrease was accounted for by water transfer.

Since most of the decrease after "holding" had usually occurred within 15 min., and since prolonged holding was tedious, this period was adopted as a routine. Data from 23 intact sheep show that the haematocrit did not decrease in one, in 3 it fell by 3-5 p.c. of the initial value, in 6 by 6-10 p.c., in 7 by 11-15 p.c., in 5 by 16-20 p.c., and in one by 24 p.c., the mean decrease being 12 p.c. In 8 splenectomized sheep, it decreased in 4 by 1 p.c., in one by 2 p.c., in two by 3 p.c., and in one by 4 p.c., the mean decrease being 2 p.c. Thomson *et al.* (1946), who also used the term "holding", had observed decreased jugular haemoglobin values after "holding", but they did not examine the effect in splenectomized sheep.

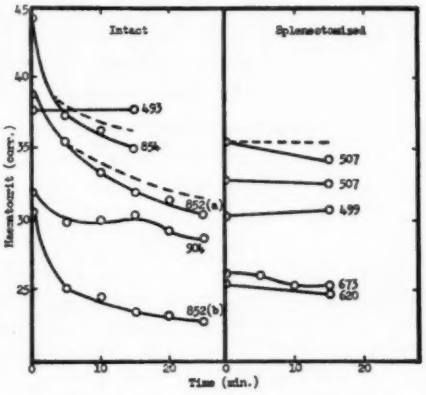

Fig. 2. Response of jugular haematocrit to quiet restraint ("holding") in intact and in splenectomized sheep. 852(b): Haematocrit reduced by a series of bleedings and plasma replacements. The broken lines in this and other figures are drawn through haematocrit values adjusted for water transfer where appropriate.

Because water transfer plays no important or constant part, the effect does not appear to be due to immobility *per se*, and because it is virtually abolished by splenectomy, it is clearly dependent upon the spleen. We therefore regard "holding" as due to an effect of the allaying of fear, i.e. of psychological tranquillization, upon the spleen.

Attempts were made to determine if the fall of jugular haematocrit is accompanied by increased numbers of red cells in the splenic region. For this purpose sheep were prepared with ^{51}Cr-labelled cells, and were used when constant counts had been reached over the spleen. The surface-counting technique was not very satisfactory in sheep subjected to "holding", perhaps because the cumbersome apparatus and necessary presence of other persons disturbed the animals. The most impressive results were obtained with a sheep which had struggled a little to avoid capture, and whose counts at the beginning of observations was consequently low (see later); from initial values about 2200/min. counts gradually increased during 15 min. "holding" to values about 4000/min.

Effect of Pentobarbitonum anaesthesia upon haematocrit and external count.

The effect of PBS anaesthesia upon jugular haematocrit was examined. As shown in Fig. 3, in intact sheep haematocrit fell rapidly at first and then less rapidly, similar to that after "holding" in typical sheep, but more regularly and more steeply during the first 5 min. Minimum values, mostly between 22 and 29, were reached within 20-30 min., and were maintained for a variable period. In some sheep evidence of water transfer under the influence of PBS was obtained, but this was sufficient to account for only a small part of the haematocrit decrease. In splenectomized sheep, haematocrit decreased only slightly

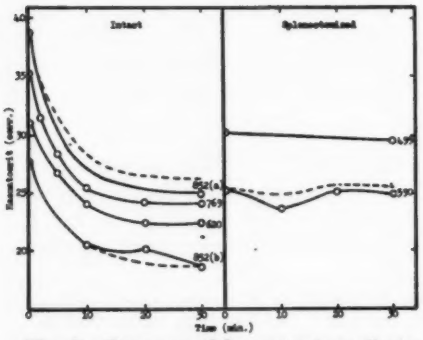

Fig. 3. Response of haematocrit to Pentobarbitonum anaesthesia in intact and in splenectomized sheep. 852(b): Haematocrit reduced by a series of bleedings and plasma replacements.

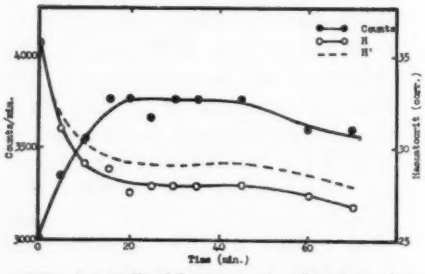

Fig. 4. Fall of haematocrit after Pentobarbitonum, with evidence of increased numbers of red cells in spleen, in sheep prepared with ^{51}Cr-labelled cells.

TABLE 1.

Comparison of effect of "holding" and of Pentobarbitonum (PBS) anaesthesia upon jugular haematocrit.

Sheep No.	Jugular haematocrit			Decrease (p.c.)		
	Initial (a)	"Holding" (b)	PBS (c)	(a-b)/a	(b-c)/b	(a-c)/a
Intact						
493	37·8	37·9	25·2	0	34	33
491	35·7	34·0	24·5	5	28	31
790	27·6	25·4	24·9	8	2	10
852(a)	38·7	33·7	24·9	13	26	36
*852(b)	27·7	24·7	18·7	11	24	32
489	39·2	34·0	28·8	13	15	27
495	35·6	30·1	22·0	15	27	38
574	35·3	29·7	27·4	16	8	22
191	35·1	29·4	28·7	16	2	18
488	34·9	29·1	22·2	17	24	36
496	36·8	29·5	22·3	20	20	39
751	35·2	26·6	24·6	24	6	30
Splenectomized						
620	32·6	—	33·3	—	—	− 2
499	30·2	—	29·5	—	—	2
590	25·8	—	24·4	—	—	5
590	22·4	—	22·5	—	—	0
590	25·1	—	24·9	—	—	1

* Haematocrit reduced by a series of bleedings and plasma replacements.

during anaesthesia, and usually this could be accounted for by haemodilution through transfer of water into the plasma.

The effects of "holding" and of PBS anaesthesia in 11 intact sheep are compared in Table 1. These sheep exhibited a wide range of response to "holding", the decrease of haematocrit ranging from 0-24 p.c. of the pre-"holding" value. At the conclusion of "holding" for 15 min. each sheep was then anaesthetized with PBS, and values of haematocrit were again determined after 30 min. The subsequent decrease varied greatly, ranging from 2-34 p.c. of the "holding" value. Significantly, an inverse relationship often occurred between the two values ($r = -0{\cdot}42$; $y = 27{\cdot}8\text{-}0{\cdot}77x$, where y = p.c. decrease of H after PBS, from "holding" values, and x = p.c. decrease after "holding", from initial value). This suggests that sheep which had responded well to "holding" usually had little capacity to respond further to PBS, whereas those which had responded poorly to "holding" were capable of relatively large responses after PBS. In three splenectomized sheep anaesthetized with PBS, haematocrit fell not at all or only slightly (5 p.c.).

By using sheep which had been prepared by ^{51}Cr-labelled cells and had reached stable counts over the splenic region, it was shown that the decrease of jugular haematocrit after induction of anaesthesia by PBS was accompanied by a reciprocal increase of gamma radiation over the splenic region, indicative of increased accumulation of red cells (Fig. 4).

Effect of psychic stimulation upon haematocrit.

Sheep were isolated as completely as possible from external sensory stimulation by placing them in crates in a remote quiet room. A polythene intravenous cannula was established in a jugular vein, and was led to the exterior. This arrangement permitted the withdrawal of samples of jugular blood without the animal's knowledge of the concealed operator's presence. After some hours, or on the next day, when the animal had settled down, preliminary blood samples were withdrawn through the cannula. The door of the room was then opened by another person, who entered the room, closed the door and stood quietly. The concealed operator outside the room again withdrew samples one minute, and again 15 min., after his colleague had entered the room.

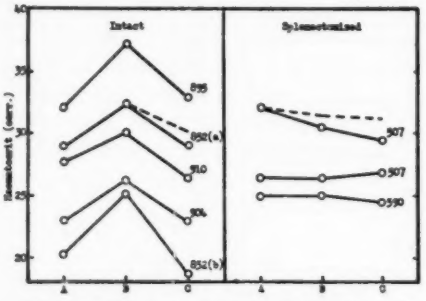

Fig. 5. Comparison of the effect of psychic stimulation upon haematocrit of intact and of splenectomized sheep maintained in solitude. Jugular blood samples were removed by a concealed operator through in-dwelling catheters as follows: A, before entry of observer; B, one minute after entry; C, 15 min. after entry. 852(b) : Haematocrit reduced by a series of bleedings and plasma replacements.

In splenectomized sheep, usually no change of haematocrit occurred; when small changes were observed, they were explicable in terms of transient water transfer (Fig. 5). In intact sheep, one minute after entry into the room the haematocrit increased by from 10 p.c. to almost 25 p.c. of the "resting" value, but usually had returned to near the resting value by the end of 15 min., in the absence of further stimulation. One intact sheep showed almost no response to room entry, or even to being approached and bled by syringe and needle, and thus behaved like a splenectomized sheep.

The technical difficulty of maintaining the heavy, bulky scintillometer in the necessary contact with the animal, without the presence of operators, did not permit application of the ^{51}Cr technique to this phase of the investigation.

Effect of exertion upon haematocrit and external count.

In this series blood samples were collected initially, after "holding" for 15 min., after chasing for 1 min., and after chasing for 8 min., at which stage the sheep were panting. As a result of "holding", haematocrit decreased by up to 19 p.c. of the initial value. The result of chasing for 1 min. was to restore the haematocrit to near the initial level, and even after chasing for 8 min. the subsequent increase was relatively small (Fig. 6). These effects were not dependent upon a high total red cell mass, for they were also obtained when this had been lowered considerably by repeated bleedings and plasma replacements

(compare 852a and 852b). In splenectomized sheep, haematocrit increased little or not at all after chasing for 1 min. or for 8 min.; indeed, it decreased somewhat after the more severe exertion to an extent greater than could be accounted for by evidence of water transfer.

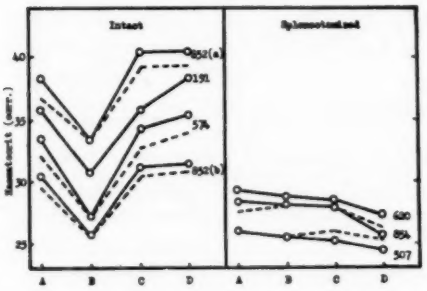

Fig. 6. Comparison of effects of "holding", mild exertion and severe exertion upon haematocrit of intact sheep and of splenectomized sheep. Samples were collected: A, when the sheep was first approached; B, after "holding" for 15 min.; C, after chasing for 1 min.; and D, after chasing for 8 min. 852(b): Haematocrit reduced by a series of bleedings and plasma replacements.

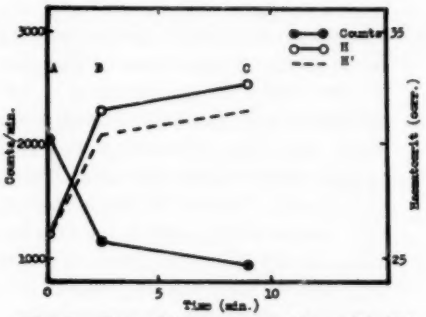

Fig. 7. Rise of haematocrit after mild (B) and severe (C) exertion with evidence of loss of red cells from spleen, in sheep prepared with ^{51}Cr-labelled cells.

By using intact sheep prepared with ^{51}Cr-labelled cells, it was shown that the increase of jugular haematocrit after mild and severe exertion was accompanied by decreases of count over the splenic region (Fig. 7).

Effect of intravenous adrenaline upon haematocrit and external count.

Intravenous injection of 1·5 ml. of the 1:1000 solution, corresponding to approx. 30 μg./kg., was followed within 15-30 sec. by severe transient tachycardia (increase from normal 60-70 to 200 or more/min.), and hyperpnoea. These effects were accompanied by sharp increases of jugular haematocrit, which reached maxima within 2-3 min., and then began to return more slowly to the initial levels. In some sheep the maximum values were maintained for several minutes before the decrease began. Changes in plasma SG, indicating water transfer, occurred in some sheep but not all; when it occurred it was sufficient to explain only a small part of the haemoconcentration in intact sheep. In splenectomized sheep, the same effects upon heart and respiration occurred, but haematocrit increased only slightly, and usually most of the small increase could be accounted for by water transfer (Fig. 8).

Smaller amounts of adrenaline had much less effect upon heart, respiration and plasma SG, but were still capable of causing considerable haematocrit

increases; thus, in a sheep in which 30 μg./kg. was followed by 30 p.c. increase of haematocrit above the initial value, 2 μg./kg. was followed by 12 p.c. increase.

As a routine, sheep were "held" for 15 min. to reduce the haematocrit to levels more characteristic of the particular sheep when unexcited. Adrenaline (1·5 ml. of the 1:1000 solution) was then injected, and blood samples were withdrawn after 2 min. and 5 min. Out of 18 sheep used in this experiment, eight responded only moderately to "holding", the decrease of haematocrit being 3-10 p.c. of the initial values; the other 10 responded better, the decrease being 11-22 p.c. After adrenaline, the increase

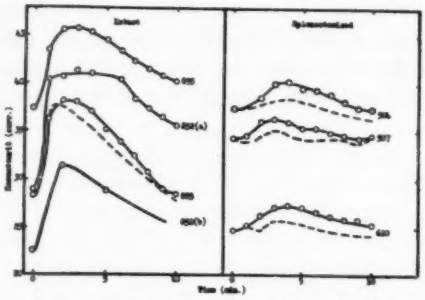

Fig. 8. Response of haematocrit to adrenaline (32 μg./kg.) injected i.v. into intact and splenectomized sheep, after "holding". 852(b): Haematocrit reduced by a series of bleedings and plasma replacements.

TABLE 2.

Increase of jugular haematocrit after intravenous adrenaline.

Sheep No.	Jugular haematocrit			p.c. Decrease due to "holding"	p.c. Increase after adrenaline
	Initial (a)	"Holding" (b)	Adrenaline (c)		
Intact					
499	35·5	33·1	35·9	7	8
496	35·8	32·9	37·0	8	12
493	40·7	39·5	45·2	3	14
495	35·9	31·4	37·2	13	18
904	29·4	26·4	31·7	10	20
895	40·7	37·2	45·6	9	23
491	35·3	33·2	41·4	6	25
519	38·8	33·3	41·5	14	25
247	40·7	33·5	44·9	18	34
885	30·8	28·3	38·1	8	35
574	33·3	30·2	40·8	9	35
488	36·1	29·9	41·1	17	37
489	37·5	33·2	46·0	11	39
883	34·5	28·8	40·2	17	40
852(a)	34·9	28·9	41·0	17	42
*852(b)	25·9	22·4	31·2	14	39
921	34·4	30·2	45·7	12	51
854	39·0	30·6	46·5	22	52
751	32·6	28·4	44·9	13	58
Splenectomized					
499	33·3	34·2	34·7	−3	1
226		32·3	33·7		4
507	35·5	34·2	36·0	4	5
590	26·5		25·4		
620	30·1		30·7		

* Haematocrit reduced by a series of bleedings and plasma replacements.

above the "holding" values ranged from 8-58 p.c. (mean, 32 ± 14). Usually, poor haematocrit response to adrenaline was associated with poor response to "holding", and *vice versa*; but in some sheep which responded poorly to "holding", haematocrit increased considerably after adrenaline. The correlation coefficient $r = +0{\cdot}62$, the regression equation being $y = 10 + 1{\cdot}8x$, where y = p.c. increase from the "holding" value after adrenaline and x = p.c. decrease from the initial value after "holding" for 15 min. In five splenectomized sheep, adrenaline injection was followed by no increase or only minor increases of haematocrit (Table 2).

Similar findings were observed after i.v. injection of adrenaline to sheep under PBS anaesthesia. This procedure was not without hazard, and some sheep so treated died. In three intact sheep, 30 min. after induction of anaesthesia haematocrit had decreased by 19-32 p.c. of the initial value; after adrenaline, it rose sharply to levels 64-66 p.c. greater than the anaesthetic levels, or 13-34 p.c. greater than the initial levels. In two splenectomized sheep similarly anaesthetized, haematocrit decreased not at all in one, and in the other by only 4 p.c. of the initial value; after adrenaline it increased only slightly, and then only to near the initial levels.

The use of conscious intact sheep prepared with ^{51}Cr-labelled red cells showed that the increased jugular haematocrit after adrenaline was mirrored almost exactly by decreased radiation over the spleen, indicating loss of red cells from the area beneath the scintillometer. A typical experiment is illustrated in Fig. 9, which also shows that when surface counts and jugular haematocrit had returned almost to their initial values, stimulation by suddenly slapping and pricking the animal's head was followed almost immediately by sharp rise of haematocrit and by simultaneous inverse fall of radiation count.

The sharp fall of splenic count after adrenaline i.v. during anaesthesia, and the subsequent recovery to higher values, are illustrated by sheep 885 in Fig. 1. This sheep had been deeply anaesthetized with PBS for 25 min. before injection of labelled cells, and therefore demonstrates incidentally that continuous displacement of red cells in the spleen by red cells in the circulating blood occurs almost as readily during anaesthesia as during consciousness.

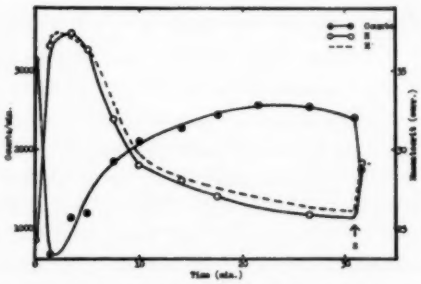

Fig. 9. Inverse relationship between haematocrit response after adrenaline (32 µg./kg., i.v.) and gamma radiation count over splenic region, in an intact sheep prepared with ^{51}Cr-labelled red cells. At S, when haematocrit had almost returned to the original level, the sheep was suddenly stimulated by cutaneous pricks about the head.

Effects of subcutaneous adrenaline upon jugular haematocrit.

Thomson *et al.* (1946) failed to detect increased concentration of haemo-

globin in the jugular blood of two sheep which had received 1-1·5 ml. of 1:1000 adrenaline subcutaneously, an amount which caused "an immediate increase in the capillary blood-sugar level and a rise in the respiration and pulse rates", and they stated that "the absence of an effect with adrenaline increases our doubt as to whether fluctuations in the size of the small spleen of sheep could give effects commensurate with those observed". In our experiments, three sheep were "held" for 15 min. to reduce the effects of capture upon haematocrit, and thereby to make it easier to observe the effect of adrenaline upon it. As a result of "holding", the haematocrit fell to levels 10-12 p.c. below the initial levels. Adrenaline (1·5 ml. of the 1:1000 solution) was injected subcutaneously, and "holding" of the animals was continued in order to facilitate the periodic venipunctures. In two sheep, heart and respiratory frequencies did not increase during the succeeding 25-30 min.; the haematocrit also did not increase, but on the contrary gradually decreased by 3-5 p.c. of the "holding" value. In the third sheep, both heart and respiratory frequencies began to increase 2 min. after the injection, from the "holding" values 72 and 40, respectively, to 126 and 140 after 25 min. Haematocrit also began to rise more or less steadily after 5 min., and after 30 min. had slowly climbed to 18 p.c. above the "holding" level. This sheep resented the continued restraint, and it is possible that some of the observed effects were due to excitement. The response of these sheep is in sharp contrast to those injected intravenously with adrenaline.

Effect of intravenous nor-*adrenaline upon jugular haematocrit and external count.*

The effects of equivalent amounts of *nor*-adrenaline and of adrenaline upon jugular haematocrit, and upon the gamma radiation counts of sheep prepared with ^{51}Cr-labelled red cells, were virtually identical and are not illustrated.

Direct observation of effect of adrenaline upon the spleen.

Direct observations were made upon the effect of adrenaline upon the sheep's spleen, both *in situ* and *in vitro*.

Contraction of spleen in situ.—Under PBS anaesthesia, the spleen was freed from its ligaments and peritoneal attachments, and was brought as far outside the laparotomy incision as the short splenic pedicle permitted without excessive torsion or stretching. The spleen, which was very dark, bluish and swollen, was 19 cm. long, 15 cm. wide, and 5-6 cm. thick. Less than 2 min. after intravenous injection of adrenaline (32 μg./kg.), contraction became observable, first at the edges, which quickly assumed a relatively sharp outline, and the colour eventually became paler and mottled. Three minutes after injection, the length had decreased to approximately 13 cm., the width to 10 cm., and the thickness to about half the initial, i.e. the volume decreased to 20-25 p.c. of the volume before adrenaline. After another minute or so the spleen began to swell again,

and seven minutes after injection the edges had lost much of their sharpness. After 16 min. it was still partly contracted, but by 20 min. it had virtually returned to the original size. Some unavoidable stretching of the pedicle, and consequent interference with circulation, cannot be totally excluded. The animal was then bled out, still under anaesthesia, and when anoxic gasping commenced the spleen again began to contract very noticeably, and finally decreased to 12 cm. long, 7·5 cm. wide, and about 2 cm. thick, or approximately one-eighth of the initial volume.

Contraction in vitro. The ligated spleen of sheep No. 488 (BW = 37·5 kg.), removed under PBS anaesthesia, weighed 519 gm., or 1·38 p.c. of the BW. The jugular haematocrit at the moment of ligation was 29, having increased to within 13 p.c. of the initial value, or 4 p.c. of the "holding" value (see later). Cannulae were introduced into the splenic artery and vein, and perfusion was carried out at the animal's arterial blood pressure and at 38° C., with Tyrode solution equilibrated with 5 p.c. CO_2 in O_2, in a bath of the same fluid. The spleen gradually relaxed until after 30 min. it weighed 791 gm. During perfusion the outflow rate increased from 160 ml./min. initially to 200 ml./min., and after the first few minutes the effluent was only faintly tinged with red cells. After a total perfusion period of 36 min., 5 ml. of 1:1000 adrenaline solution (≡ 5 mg. adrenaline) were injected into the inflow perfusion tube. The spleen quickly began to contract, and large quantities of thick, dark blood passed along the outflow tube, in marked contrast to the almost clear fluid preceding it. Within 3 min. outflow ceased, and the weight of the spleen had decreased to 132 gm., indicating a loss of 387 gm. By haemoglobin estimation of the effluent, it was calculated that 260 ml. of red cells, equivalent to mean haematocrit = 73 in the splenic blood, had been ejected from the spleen.

An attempt to perfuse the spleen of a sheep which had been exsanguinated when under PBS anaesthesia, and whose spleen had undergone agonal contraction, was unsuccessful; no perfusate dripped from the effluent cannula during 2 hr., but the weight of the spleen increased from 99 gm. to 180 gm.

The size of the spleen in sheep.

In the conscious resting sheep, free from alarming stimuli, the ratio of spleen weight to BW should provide a good estimate of the potentiality of its spleen as a red cell reservoir. However, since direct observation in the conscious animal could not be used, it was necessary to make indirect estimates.

(a) *The spleen in the exsanguinated sheep.* The spleens were examined in 20 sheep of mean live weight 45 ± 4·5 kg., slaughtered at an abattoir. The mean measurements were: length 12·1 ± 1·15 cm., breadth 8·0 ± 0·69 cm., maximum thickness 2·3 ± 0·27 cm., volume by displacement 83·5 ± 14·5 ml., SG 1·09 ± 0·05, and weight 90·8 ± 12·5 gm. The ratio of spleen weight to BW was not strikingly less than that reported in dogs, the mean (as percentage) being

0·204 ± 0·031 and the range from 0·15 to 0·27; indeed, one-fifth of the values were greater than the value of 0·22 p.c. found in an exsanguinated dog by Barcroft and Stephens (1927-28) and as a mean value in three such dogs by McCance and Widdowson (1955).

(b) *The spleen removed under PBS anaesthesia.* In sheep, the spleen is related to the diaphragm, to which it is attached over the anterior third of its parietal surface; it is also attached over the anterior half of its visceral surface to the rumen, and over its base to the left crus of the diaphragm, under the last two ribs. It is therefore not easily accessible, and considerable blunt dissection and handling is necessary before the very short pedicle can be ligated, and the organ ablated. The sheep were anaesthetized by intravenous injection of PBS; laparotomy began about 10 min. after the injection, and ligation of the splenic pedicle was usually performed about 10 min. later. All spleens were greatly enlarged as compared with those of slaughtered sheep; the surfaces were convex, the edges and corners rounded, they were easily ruptured by careless handling, and were engorged with dark blood. The dimensions of the largest spleen measured were (sheep No. 519) length 18·6 cm., width 13·7 cm., thickness 5·5 cm., and weight 605 gm., corresponding to 1·33 p.c. of BW. In Table 3 particulars are given of 17 spleens in which relatively small loss of blood occurred

TABLE 3.

Weight of spleens removed under Pentobarbitonum anaesthesia, in relationship to jugular haematocrit at moment of ligation.

Sheep No.	Body weight (a) (kg.)	Spleen weight (b) (gm.)	Ratio of b:a (p.c.)	p.c. by which haematocrit at moment of splenic ligation differed from			
				Initial value		"Holding" value	
				−	+	−	+
496	33·5	256	0·76	3			6
497	35·0	320	0·91				
488	37·5	519	1·38	13			4
507	39·0	467	1·20	2			25
504	39·5	554	1·40		4		34
247	40·5	585	1·44	12		5	
493	41·0	474	1·16				
568	42·5	295	0·69	9			
261	42·5	500	1·18				
910	43·0	560	1·30				
495	44·0	406	0·92		1		6
226	45·0	440	0·98	5			1
489	45·0	575	1·28		11		27
854	45·0	600	1·33		9		17
519	45·5	605	1·33				
885	46·0	561	1·22				
822	46·5	645	1·39				

during ablation. The spleen weights ranged from 256 to 645 gm., the mean being 492 ± 115 gm.; the ratio of spleen weight to BW ranged from 0·69 p.c. to 1·44 p.c., the mean being 1·17 p.c.

(c) *Estimated size of the spleen in the conscious sheep.* During splenectomy operations it was sometimes observed that immediately after completion of the operation, while the animals were still deeply anaesthetized, the jugular haematocrit had risen from the low levels associated with PBS anaesthesia to levels characteristic of the conscious animal, and that the haematocrit of the blood in the splenic vein was then almost twice that of the jugular blood. Investigation showed that the rise of jugular haematocrit occurs during the stage of blunt dissection and separation of the spleen. In the instance illustrated (Fig. 10), jugular haematocrit, which had remained at levels around 30 during the stages of laparotomy, suddenly increased about 30 p.c. when the spleen was freed by blunt dissection from its attachments to diaphragm and rumen. This was about 8 p.c. greater than when the first sample was collected before the operation. The ablated spleen in this instance weighed 600 gm., or 1·33 p.c. of the BW.

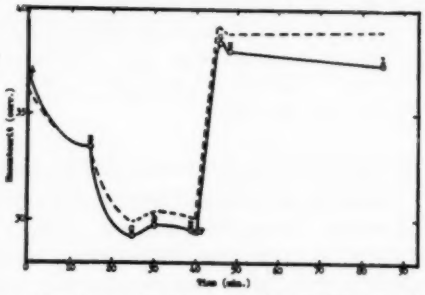

Fig. 10. Rise of jugular haematocrit in the Pentobarbitonum-anaesthetized sheep when its engorged spleen was separated from its attachments during splenectomy. A, initial; B, after 15 min. "holding"; PBS administered; C, 10 min. after PBS; D, 15 min. after PBS, skin incision commenced; E, 24 min. after PBS, peritoneum entered; F, 25 min. after PBS, laparotomy completed, spleen visible, separation commenced; G, 31 min. after PBS, separation completed; H, 33 min. after PBS, spleen ligated and removed; I, 70 min. after PBS, skin sutures completed, animal still deeply anaesthetized.

These observations suggest that trauma and handling, inseparable from splenectomy in the sheep, usually stimulate some degree of splenic contraction, leading to the ejection of sufficient cell-rich blood into the general circulation to cause an increase in jugular haematocrit. It therefore seems reasonable to assume that, when jugular haematocrit has returned to near the "pre-anaesthetic" level, the weight of the ablated spleen is not far removed from what it was when the animal was conscious.

The data in Table 3 have been used to provide estimates of the mean ratio of spleen weight to BW in sheep (a) moderately excited, as when first seized, and (b) relatively tranquil, as a result of "holding". For the first estimate, the ratios based upon sheep in which jugular haematocrit had returned to within 5 p.c. (mean = 1 p.c.) of the initial value (sheep Nos. 496, 507, 504, 495 and 226) provide a mean estimate of spleen weight/BW = 1·05 p.c. For the second estimate, the ratios based upon sheep in which jugular haematocrit had returned to within 5 p.c. (mean = 0 p.c.) of the "holding" value (sheep Nos. 488,

247, and 226) provide a mean estimate of spleen weight/BW = 1·27 p.c.; this is somewhat higher than estimate (a), although not significantly so. The pooled mean is 1·15 p.c., i.e. 5·75 times the mean ratio of spleen weight/BW found in slaughtered exsanguinated sheep. On the assumptions made, it therefore appears that in conscious sheep not excessively excited the weight of the spleen may be on the average about six times that observed in exsanguinated cadavers.

If the mean weight of the spleen in conscious but not unduly alarmed sheep is therefore taken as 11·5 gm./kg., and its mean weight in the exsanguinated slaughtered sheep as 2·0 gm./kg., the amount of blood contained in it may be taken as of the order of 9·5 gm./kg. BW. If the mean haematocrit of splenic blood is conservatively taken as 55, the spleen therefore contains 5·23 ml. red cells and 4·27 ml. plasma, per kg. BW. By means of T-1824 and ^{51}Cr, mean estimates of PV = 44·6 ml./kg., RCV = 20·2 ml./kg., and BV = 64·8 ml./kg. have been obtained (to be published). Hence, on the basis of these calculations, the spleen of the average unalarmed sheep contains 9·5/64·8 = 14·7 p.c. of the total BV (approx. one-seventh) and 5·23/20·2 = 25·9 p.c. of the total RCV (approx. one-fourth).

Cell-concentrating mechanism in the sheep's spleen.

Hahn *et al.* (1942) found no significant difference in the total cell mass of dogs when determined 4-5 min. after injecting isotope-labelled red cells and again 3 min. after an immediately subsequent injection of adrenaline; they therefore concluded that most of the red cells in the spleen of the conscious dog are in "active circulation" and are not "sequestered" in the sense attributed to Barcroft *et al.* (1925). The labelled-cell uptake curves in Fig. 1 show that in sheep, although about half the red cells in the spleen may be displaced by circulating red cells within 4-5 min., a significant proportion remains in the spleen for much longer periods. The counts obtained in sheep Nos. 895 and 904 in Fig. 1 were used to construct a mean curve A (Fig. 11) which shows that after 2, 4, 5, 6, 8, 10, 15 and 20 min. counts over the spleen had reached 28, 49, 56, 60, 71, 78, 90 and 94 p.c., respectively, of the maximum. This may be interpreted as indicating that in such sheep at any moment of time, 44 p.c. of the red cells then present had been there for at least 5 min., 22 p.c. for at least 10 min., 10 p.c. for at least 15 min. and 6 p.c. for at least 20 min. From this curve, the rate of displacement of splenic red cells by circulating red cells can be obtained by measuring the tangents at various points. These values, corresponding to dN/dt, when plotted against time, yield the curve B in Fig. 11, and demonstrate that the rate of displacement progressively decreases. This suggests differences in the ease with which splenic blood is displaced in various parts of the spleen. The most likely differences are as between large vessels, small vessels, capillaries and the concentrated blood in sinuses.

The findings with sheep 885 (Fig. 1), which had been anaesthetized with PBS for 24 min. when labelled cells were injected, were essentially similar to those with the two conscious sheep, except that counts over the spleen increased somewhat more slowly, having reached after 5, 10, 15 and 24 min. 50, 66, 73 and 75 p.c. of the potential maximum as revealed by use of adrenaline.[1] The apparent flattening of this curve, taken with evidence obtained during blood volume determinations in sheep that had been "held" (to be published), suggests that when the spleen is very dilated, prolonged stagnation ("sequestration") of red cells may occur in some areas or structures.

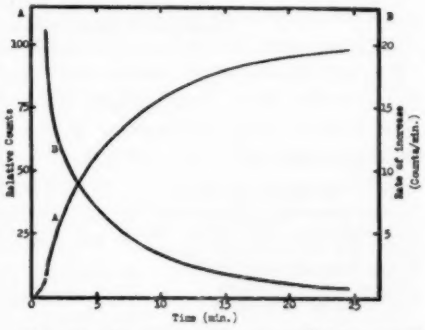

Fig. 11. Increasing gamma radiation count over splenic area after intravenous injection of ^{51}Cr-labelled red cells (Curve A), compared with decreasing rate of increase (Curve B). Data derived from the means of the values for sheep 895 and 904 (Fig. 1).

During splenectomies the splenic pedicle was routinely ligated immediately after separation of the spleen from its peritoneal and ligamentous attachments. Under these conditions, when the jugular haematocrit, as stated earlier, had usually increased considerably, the haematocrit of blood from the splenic vein was much higher than that in the jugular blood. However, when the interval between splenic separation and pedicle ligation was deliberately prolonged for 15 min., the haematocrit value of splenic-vein blood was sometimes less than that in the jugular vein (sheep 910 and particularly 885, Table 4). This can only mean that in those sheep red cells were being removed from the perfusing afferent blood and that the depleted blood rejoins the unconcentrated blood before it leaves the spleen. The high values for splenic-vein haematocrit in sheep 358 and 882 are presumed to be due to the difficulty of tying the pedicle ligature in sheep without touching or compressing the spleen, and thereby stimulating contraction.

Estimates of the degree of red cell concentration in the storage sites, i.e. sinuses, were made in the sheep summarized in Table 4 by cannulating the artery and vein, after the splenic-vein blood had been allowed to flow out. Adrenaline diluted in saline, usually 5 ml., was then slowly injected into the arterial cannula and the blood ejected through the vein was collected. This procedure was repeated until no further response was obtained. Haematocrits

[1] At this point the sheep received adrenaline i.v. to contract the spleen; counts rapidly decreased more than 50 p.c. in 2 min., then began to rise quickly and within 18 min. had reached a plateau which was assumed to represent the maximum. Two hours later this sheep was again anaesthetized, when the counts rose to approximately the same level to which they had risen after recovery from adrenaline.

TABLE 4.

Jugular and splenic-vein haematocrits during splenectomies.

	Sheep No.				
	885	895	358	882	910
Jugular haematocrit					
Initial	31·6	32·0	—	32·7	28·0
After "holding" (15 min.)	26·9	25·1	—	28·1	24·9
After anaesthesia (20-30 min.)	17·7	22·5	—	22·4	22·0
Immediately after spleen "freed"	28·2*	30·5*	n.s.	31·1	27·5
10-15 min. later; pedicle ligated	—	—	27·5	25·3	27·4
Splenic haematocrit‡					
Splenic vein (outflow)	18·3	30·6	41·5	67·2	25·3
Ejected by adrenaline	71-73	55·0†	72-86	89-93	53-81

* Spleen ligated and removed immediately after "freeing".

† Only one sample collected.

‡ The higher values may be slightly over-estimated through less efficient packing, since the TPCF employed (0·97) had been determined for the range 24-53 (Hodgetts, 1959); however, the extreme value 93·2 for sheep 882 (85·7 before adjustment for adrenaline) fell to only 92·4 (84·8 before adjustment) after a further 60 min. at 4756 *g*.

were determined on the separate fractions (30-150 ml.), and were adjusted to allow for the volume of the injected adrenaline solution, assuming that this all appeared in the fraction. Since the "adjusted" haematocrits of the separate fractions from each spleen were very similar, the assumption appears to be valid. The haematocrit of blood in the smaller vessels or sinuses may therefore be of the order of 70-90. Frequently the later fractions exhibited a slight degree of haemolysis, indicating increased cell fragility. This was also observed in dogs by Penrod (1952), and probably is the result of prolonged hypoxia of cells retained for long periods in engorged sinuses.

DISCUSSION.

The evidence strongly suggests that the changes of jugular haematocrit described are inversely related to changes in the size of the spleen, i.e., to changes in its degree of filling. The importance of the spleen as a regulator of red cell concentration in sheep derives on the one hand from its relative size and on the other hand from its high degree of red cell concentration as compared with circulating blood. The ovine spleen is of the same order of size, relatively to the body weight, as in dogs, and is therefore not the relatively small organ suggested by Thomson *et al.* (1946). Furthermore, the evidence suggests that in the conscious sheep, not excessively alarmed, it may weigh more than 1 p.c. (in one instance, 1·4 p.c.) of the body weight. We could find no comparable data for dogs, except the record of 1·8 and 2·0 p.c. (Barcroft and Stephens, 1927-28) for denervated venously-ligated spleens, which therefore may have dilated beyond normal limits. However, size *per se* has significance only

if at the same time a mechanism exists for concentrating red cells in the splenic blood. Mathematical treatment shows that the potential effectiveness of the spleen as a regulator of extrasplenic red cell concentration will increase as the disparity between splenic and extrasplenic haematocrit values increases. We found that the haematocrit of the pulp blood might reach 70-90, whereas the mean jugular haematocrit of our sheep is of the order of 36 under average conditions or 31 when tranquillized by "holding" (see Table 2). The ratio of splenic to jugular haematrocrits in sheep may therefore be 2-3, whereas in dogs, whose peripheral haematocrit is considerably greater than in sheep, Barcroft and Poole (1927-28) found this ratio to be of the order 1·3-1·7. This is probably the main reason why the proportionate decrease of jugular haematocrit during PBS anaesthesia and the proportionate increase after adrenaline injection are greater in sheep than has been reported in dogs. Our calculations that the spleen of the average unalarmed sheep may contain about one-seventh of the total blood volume and one-fourth of the total red cell volume may be compared with those of Barcroft and Stephens (1927-28) which suggest that the dog's spleen may contain about one-fifth of the total red cells.

It is not difficult to visualize how plasma is filtered from blood during transit through the elaborate system of thin-walled perforate "sinuses" in the spleen of man and mouse (Weiss, 1957), but it is fairly certain, from the instances of lower haematocrit values in the splenic vein than in the jugular vein (Table 4), that in sheep much of the separated plasma rejoins the blood before the emergence of the splenic vein, and not external to the spleen *via* the lymphatic drainage (Barcroft and Poole, 1927-28; Barcroft and Florey, 1928). In the spleen at equilibrium it may be presumed that as many sinuses are becoming engorged with concentrated red cells as are allowing their contents to pass onwards with the perfusing blood. No histological evidence for the existence of distal sinus sphincters to retain concentrated blood (Knisely, 1936) was found by Weiss (1957), nor were they observed in the transilluminated living spleen by Whipple *et al.* (1954). These authors recognized instead a vascular shunting system. Stasis or progression of blood may depend upon the action of these vascular shunts or of the larger arteriovenous anastomoses demonstrated by Prinzmetal *et al.* (1948). Relaxation of vascular shunts, and consequent extensive bypassing of the engorged sinuses, may explain our inability to wash the isolated engorged spleen free of red cells by lengthy perfusion with Tyrode solution, whereas if flow of blood through the sinuses were controlled by distal occluding muscular sphincters, these should relax after nerve severance and complete washout should be possible. The failure to perfuse the isolated anoxia-contracted spleen is less easy to explain on these grounds, since *in vivo* the adrenaline-contracted spleen begins to fill again within a few minutes: possibly in the absence of nervous control persistent spasm of arterioles occurs through failure of the *nor*-adrenaline inactivating system.

The question whether or not red cells are held "in reserve", i.e. sequestrated, in the spleen may now be considered to have lost some of its importance since the answer turns partly on the definition of sequestration and on its quantitative aspects. In sheep the scintillometer evidence clearly shows that red cells "sojourn" in the spleen for periods greatly in excess of the normal transit time through other organs. The scintillometer technique might profitably be applied to the problem of "rapid mixing" in dogs, as permitting observations with minimum emotional disturbance. Possibly in the experiments of Hahn *et al.* (1942) the manipulations and injections associated with the isotope-dilution technique may have caused splenic contraction and thus led to the evidence of almost complete exchange of circulating and splenic red cells within a period of only 5-8 min., whereas in anaesthetized dogs massive "sequestration" could be demonstrated (Hahn *et al.*, 1943). It is likely that the behaviour of the spleen in dogs, as in sheep, is essentially similar during consciousness or unconsciousness, differing only in degree. From the statistical viewpoint, the spleen is to be regarded as a dynamic reservoir of red cells, a term also used by Green (1950), and it illustrates a mechanism by which red cells in excess of the oxygen capacity demanded by the existing situation are withheld from the circulation, virtually on a rota system, for periods which are not so prolonged as to lead to excessive vitiation. Barcroft's conception of the spleen as a regulator of red cell concentration in the peripheral blood is in no essential manner weakened by the change of emphasis from static to dynamic storage.

Hahn *et al.* (1942), in rejecting the primary rôle of the spleen in jugular haematocrit rises after adrenaline in dogs, placed some importance on the variations of response as between dogs. This does not seem important to us. Many factors could contribute to this, including:

(i) individual variations in spleen size and capacity;
(ii) high initial haematocrit due to excitement; and
(iii) diminution of spleen size through transfer of blood to uterus during oestrus (Barcroft and Stevens, 1928).

The demonstration that in some instances the jugular haematocrit of sheep may increase by 25 p.c. of the initial value during excitement, or decrease by a similar proportion during repose, introduces formal difficulties in defining the normal haematocrit value, red cell count or haemoglobin concentration of sheep, since clearly these values may fluctuate by a factor approaching 2, depending upon the emotional status or consciousness of the animal at the moment when the blood samples were collected. A practical compromise likely to avoid excessively high or low values is to accustom sheep to personnel, environment, manipulations, and routine, and always to practise quietness and gentleness. The fall of haematocrit during quiet "holding", which may be regarded as a form of psychological tranquillization, suggests that pharmacological tranquillization by means of ataractic drugs may have a similar effect; and indeed this has proved to be so (to be published).

Since the completion of this investigation decrease of jugular haematocrit in sheep during anaesthesia, and increase after exercise, adrenaline, or related substances have been reported by others (Evans, 1957; Bianca, 1957).

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BIOCHEMICAL STUDIES ON *NIPPOSTRONGYLUS MURIS* INFESTATION

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SUMMARY.

Liver and jejunal metabolism has been studied in rats infested with the intestinal nematode *Nippostrongylus muris*. The aspects of liver metabolism were found to be normal, but jejunal metabolism abnormal, with enzyme preparations from infested rats.

Oxidative phosphorylation was uncoupled in mitochondria from infested jejuna and phosphorylation was severely inhibited. The uncoupling factor was shown to be calcium, which was present in inhibitory concentrations in the enzyme preparations from infested jejuna. Homogenized *N. muris* were found to contain high concentrations of calcium. Calcium was demonstrated, histochemically, to be accumulated in cells lining the parasite gut. It is considered probable that parasites homogenized inadvertently with infested jejunal mucosa supplied the calcium to uncouple oxidative phosphorylation. When calcium was chelated by EDTA, jejunal metabolism was normal in infested rats.

The significance of these results is discussed. The study suggests that *N. muris* does not interfere with the host's intermediary metabolism and thus emphasizes more strongly the possibility of disturbance of physiological mechanisms to produce the pathological signs of infestation.

INTRODUCTION.

The manner in which many gastro-intestinal parasites influence the metabolism of their hosts to produce pathological signs is obscure. This study was designed to determine whether parasitic infestation affects the subcellular metabolism of the host either at the site of infestation or in another tissue. Such an effect might be produced if the parasite were to deprive the host of specific nutrients or to elaborate toxic substances.

Acute infestation of rats with the jejunal nematode *Nippostrongylus muris* was chosen as the experimental subject. The known pathology of this infestation has been described by Porter (1935), Taliaferro and Sarles (1939) and Symons (1957).

Materials and Methods.

Enzyme preparations.

Rats were killed by a blow on the neck. Tissue from at least six acutely infested rats was removed rapidly, immersed in ice-cold 0·25M sucrose and combined for each enzyme preparation. Liver homogenates were prepared in 0·25M sucrose with glass homogenizers and fractionated by differential centrifugation in the cold (Schneider, 1948). Segments of jejunum were washed in ice-cold 0·25M sucrose or 0·25M sucrose + 0·003M ethylenediaminetetracetic acid (EDTA) as indicated in the text; washed internally with 10 ml. of the same medium under pressure from a syringe to eliminate ingesta and as many worms as possible; minced with scissors; homogenized in a loose-fitting glass homogenizer to strip the muscle and peritoneum fairly effectively from the homogenized mucosa; centrifuged at 40-60 *g.* for two minutes to remove strips of muscle and large fragments. The supernatant was re-homogenized in a close-fitting glass homogenizer to provide the basic homogenate for enzyme preparations and further fractionation by the Schneider (1948) procedure. Mitochondria were re-suspended in 0·25M sucrose or 0·25M sucrose + 0·003M EDTA as indicated.

Hexokinase was prepared from yeast by the Cross *et al.* (1949) modification of the method of Berger *et al.* (1946). The fraction precipitating between 25 p.c. and 40 p.c. by volume of ethanol was dissolved in 1 p.c. glucose and stored frozen. The optimal addition for oxidative phosphorylation and glycolysis was determined by assay in the appropriate system.

Reagents.

Adenosine triphosphate (ATP), adenosine-5-monophosphate (AMP), coenzyme I (CoI), nicotinamide, reduced glutathione (GSH), fructose-1:6-diphosphate (HDP) glucose, pyruvate, citrate, α-oxoglutarate, L-glutamate, succinate, L-malate, octanoate, ethylenediaminetetracetic acid (EDTA) and inorganic reagents were obtained commercially, checked for inhibitory impurities, and purified where necessary. Cytochrome c was prepared by the method of Keilin and Hartree (1937) but dialyzed against distilled water. Cytochrome c was also obtained commercially. Substrates were neutralized and used as sodium salts.

Estimations.

Oxygen uptakes and CO_2 evolution were measured with Warburg manometers.

The standard system used for respiratory experiments was: AMP 0·001M; $MgSO_4$ 0·0067M; KCl 0·025M; NaK phosphate buffer, pH 7·4, 0·0133M; cytochrome c 0·00001M; substrate 0·01M; enzyme in 0·25M sucrose or 0·25M sucrose + 0·003M EDTA as specified; water to 1·5 ml. final volume; 0·1 ml. 20 p.c. KOH in centre well to absorb CO_2; gas phase, air; temperature, 38° C.; equilibration period, 10 minutes.

The standard medium used for glycolytic experiments was: ATP 0·001M; AMP 0·001M; KCl 0·025M; $MgSO_4$ 0·0067M; NaK phosphate buffer, pH 7·4, 0·0133M; Na pyruvate 0·0067M; $KHCO_3$ 0·02M; NaF 0·01M; CoI 0·0005M; nicotinamide 0·04M; GSH 0·00067M; fructose-1:6-diphosphate 0·01M; enzyme as indicated in text; water to 1·5 ml. final volume; gas phase, N_2:CO_2 95:5; temperature 38° C.; equilibration period, 10 minutes.

The standard medium used for oxidative phosphorylation was: AMP 0·001M; $MgSO_4$ 0·0067M; KCl 0·025M; NaK phosphate buffer, pH 7·4, 0·0133M; cytochrome c 0·00001M; glucose 0·033M; NaF 0·01M; hexokinase 0·01 ml.; water to 1·5 ml. final volume; gas phase, air; temperature, 38° C.; equilibration period, 10 minutes. Liver experiments were carried out by the zero time control method to determine phosphorus esterification. Jejunal enzyme preparations possessed so much phosphatase activity that there was a net output of inorganic phosphorus during the experiment and incubated controls were necessary. Substrate was not added to the incubated controls but enough endogenous substrate was present

to give rates of oxidation appreciable enough to make the substrate P/O ratios, obtained by differences, valid.

Nitrogen was determined by the micro-Kjeldahl method of McKenzie and Wallace (1954); inorganic phosphate by the method of Fiske and Subbarow (1925) and calcium by the method of Golby *et al.* (1957).

Dry weights were determined after extracting the sample three times with boiling ethanol:diethyl ether, 3:1 v/v, to remove lipids, drying in an oven at 100° C. for 24 hours and cooling to room temperature in a desiccator before weighing.

Histochemical methods.

Calcium was stained by the method of Dahl (1952).

RESULTS.

Liver metabolism.

Liver was selected as the tissue most likely to show defective metabolism if toxic substances were absorbed by the host as a result of infestation or if the parasite induced specific nutrient deficiencies.

Anaerobic glycolysis of fructose-1:6-diphosphate by homogenates prepared from the pooled livers of six rats at each of two stages, the seventh and tenth day, of severe infestation with *N. muris*, was compared with anaerobic glycolysis by liver homogenates prepared from normal control rats of the same age and sex, at the same time and under the same experimental conditions. Repeated experiments failed to show any significant difference in $Q^{N}_{CO_2}$ (μl. CO_2 evolved/mg. fat free dry homogenate/hour) between liver homogenates prepared from infested or control rats.

The oxidation of the tricarboxylic acid cycle intermediates, citrate, succinate and L-malate, an amino acid, L-glutamate, and a fatty acid, octanoate, by homogenate and by mitochondrial preparations of the pooled livers of groups of six rats was also studied in repeated experiments carried out on the seventh and tenth day of infestation. The Q_{O_2} (μl. O_2 taken up/mg. fat free dry homogenate/hour) of homogenate preparations and the $Q_{O_2}^{(N)}$ (μl. O_2 taken up/mg. N/hour) for mitochondrial preparations for any of the substrates was not significantly different from that of control preparations from normal rats of the same age and sex which were assayed under the same experimental conditions at the same time.

It was found similarly that the efficiency of oxidative phosphorylation by mitochondria oxidizing citrate or succinate was not different in preparations from livers of infested or control rats.

Jejunal metabolism.

Difficulty was experienced at first in preparing suitable enzymes from jejuna. The muscle coats are not easily homogenized with glass homogenizers on the one hand, but on the other, the time involved in stripping the mucous membrane from the muscle prior to homogenization is too long to preclude "ageing"

of the enzymes when at least twelve animals are used for each experiment. However, the procedure described in the Methods section was developed and found to be quite satisfactory. Muscle is removed easily and a reliable homogenate prepared in a short time.

a. *Anaerobic glycolysis.* Jejunal homogenates glycolyze fructose-1:6-diphosphate briskly and Table 1 shows that infestation does not influence the rate.

TABLE 1.

Anaerobic glycolysis by jejunal homogenates.

Animals	$Q_{CO_2}^{N_2}$ (μl CO_2 evolved/mg. fat free dry enzyme/hour)
Control	78
Infested	81

System: as in Methods; enzyme prepared in 0·25M sucrose +0·003M EDTA, 4 mg. dry weight/flask. Rats infested for 7 days. Q_{CO_2} calculation based upon CO_2 evolution for 20 minutes.
Results expressed are the means of two experiments, each with the pooled jejuna of six infested rats.

b. *Oxidative metabolism.* Infestation does appear to produce a serious disturbance of tricarboxylic acid cycle activity. The rate of citrate oxidation is increased twofold and succinate oxidation more than threefold (Table 2).

TABLE 2.

Oxidation of tricarboxylic acid cycle intermediates by jejunal mitochondria.

Additions	$Q_{O_2}^{(N)}$ (μl.O_2 uptake/mg. enzyme N/hour)	
	Control	Infested
Citrate 0·01M	133	259
Succinate 0·033M	147	474

System: as in Methods; enzyme prepared and added in 0·25M sucrose, about 1·4 mg. N/flask; rats infested for 8 days; Q_{O_2} calculation based upon oxygen uptake during 20 minutes.
Results expressed are the means of four experiments, each with the pooled jejuna of six infested rats.

c. *Oxidative phosphorylation.* The reason for this acceleration of oxidative metabolism became clear when oxidative phosphorylation was studied. Table 3 shows that the efficiency of oxidative phosphorylation is very much impaired for the oxidation of either citrate or succinate, especially succinate, by mitochondria from infested jejuna.

These results clearly indicate an uncoupling of oxidative phosphorylation with a concomitant increase in oxygen uptake due to relief from the inhibition of oxidation normally imposed by phosphorylation.

TABLE 3.

Oxidative phosphorylation by jejunal mitochondria.

Additions	O_2 uptake (μ atoms)			O_2 uptake (substrate -endogenous)		P esterified (μ moles P endogenous incubations-substrate incubations)		P/O (μ moles P esterified/ μ atom O_2 used)	
	Control	Infested		Control	Infested	Control	Infested	Control	Infested
		20 min.	30 min.						
—	2·1	3·3	4·1	—	—	—	—	—	—
Citrate 0·01M	3·3	—	4·7	1·2	0·6	3·5	1·1	2·9	1·8
Succinate 0·033M	3·2	7·0	—	1·1	3·7	2·5	0·8	2·3	0·2

System: as in Methods; enzyme prepared and added in 0·25M sucrose, about 1·4 mg. N/flask; duration of experiment, 30 minutes except for succinate oxidation by infested enzyme which was 20 minutes.

The results expressed are the means of two experiments, each with the pooled jejuna of six infested rats.

The effect of homogenized N. muris.

It was, of course, of considerable interest to determine the cause of uncoupling of oxidation and phosphorylation. As a number of parasites remained attached to the jejunal mucosa and were incorporated into enzyme preparations, they could have been the source of the inhibitory material. To test this, homogenates were prepared from adult *N. muris*. These homogenates were then checked for their effects on oxidative phosphorylation by liver mitochondria.

The worms were collected in water by washing from jejuna, isolated by centrifugation at 500 *g.* for 10 minutes, washed by suspension in water and reisolation three times, and homogenized in water with a glass homogenizer to give a final concentration of 1 ml. packed parasites in 10 ml. Homogenates were added to Warburg flasks at 0·2 ml./flask.

Addition of adult worm homogenates to liver mitochondria oxidizing succinate resulted in an uncoupling of oxidative phosphorylation apparently similar to that observed in infested jejunal preparations (Table 4). Oxygen uptake was increased and phosphorus was not esterified. The additional oxygen uptake was not due to metabolism of the worm homogenate by liver mitochondria, nor to metabolism of liver mitochondria or succinate by the worm homogenate. Nor was the failure to esterify phosphorus only an apparent one due to phosphatase activity added or stimulated by the worm homogenate (Table 4), for if this were so, a significant output of phosphorus should have occurred upon addition of the *N. muris* homogenate in the absence of substrate. Endogenous respira-

tion by liver mitochondria was negligible. The presence of worm homogenate also blocked phosphorus esterification during citrate oxidation (Table 4). Unlike the experiments with jejunal preparations there was inhibition of oxygen uptake. The inhibition of oxidation could be quite unrelated to the failure to esterify phosphorus as the worm "homogenate" is really a most heterogeneous mixture. The term homogenate in enzymology means simply a suspension of broken cells. However, these results suggest the possibility of an uncoupling factor such as calcium ions which in low concentration block phosphorus esterification and stimulate oxygen uptake while in higher concentration they inhibit the oxidation of substrates other than succinate (Potter *et al.*, 1953; Lehninger *et al.*, 1954; Lindberg and Ernster, 1954; Ernster *et al.*, 1955). Also, calcium affects citrate oxidation differently from tissue to tissue. Gallagher, Judah and Rees (1956a) found that calcium accelerates the rate of oxidation of citrate by brain enzymes, whereas Hartman and Kalnitsky (1950) and others have shown citrate oxidation by liver enzymes to be inhibited by very low concentrations of calcium. A response to calcium similar to brain might occur with jejunal enzymes.

TABLE 4.

Effect of homogenized adult N. muris *on oxidative phosphorylation by liver mitochondria.*

Additions	O_2 uptake (μ atoms)	P esterified (μ moles)	P/O (μ moles P esterified/μ atom O_2 used)
Succinate 0·01M	5·5	10·6	1·9
Succinate 0·01M + *N. muris* homogenate	7·7	0	0
N. muris homogenate	0·5	−0·5	0
Succinate 0·01M +*N. muris* homogenate (no liver enzyme)	0·9	0	0
Citrate 0·01M	3·7	9·0	2·4
Citrate 0·01M +*N. muris* homogenate	2·0	0	0

System: as in Methods; *N. muris* homogenate prepared as in text, 0·2 ml./flask; duration of experiment, 30 minutes.

Effect of extracts of N. muris.

Aqueous extracts of homogenized adult *N. muris* were prepared as follows: a fresh homogenate was allowed to stand at 0° C. for 15 minutes and centrifuged at 45,000 *g.* for 20 minutes in a refrigerated centrifuge. A clear, yellowish supernatant was collected and added to reaction mixtures at 0·2 ml./Warburg flask.

Table 5 shows such an extract to have a similar but less severe effect on oxidative phosphorylation as the parent homogenate, completely inhibiting phosphorylation accompanying succinate oxidation and considerably reducing the efficiency of oxidative phosphorylation with both citrate and L-glutamate.

TABLE 5.

Effect of an aqueous extract of homogenized adult worms on oxidative phosphorylation by liver mitochondria.

Additions	O_2 uptake (μ atoms)	P esterified (μ moles)	P/O (μ moles P esterified/μ atom O_2 used)
Succinate 0·01M	5·7	10·4	1·8
Succinate 0·01M+ *N. muris* homogenate	7·0	0	0
Succinate 0·01M+ *N. muris* extract	5·4	0	0
Citrate 0·01M	6·7	15·0	2·2
Citrate 0·01M+ *N. muris* homogenate	2·2	−1·5	0
Citrate 0·01M+ *N. muris* extract	4·8	5·5	1·1
L-glutamate 0·01M	3·7	9·0	2·4
L-glutamate 0·01M +*N. muris* extract	3·6	5·5	1·5

System: as in Methods; worm homogenates and extract prepared as in text, 0·2 ml./flask; duration of experiment, 30 minutes.

Effect of EDTA.

The solubility in water and the nature of uncoupling further suggested that the factor concerned might be Ca^{++}. Virtual confirmation of this is shown in Table 6. Addition of ethylenediaminetetracetic acid (EDTA), which chelates calcium strongly, completely removes the uncoupling effect of worm extracts on succinate, citrate and L-glutamate oxidations.

TABLE 6.

Effect of EDTA on the inhibition of oxidative phosphorylation by extracts of Nippostrongylus muris.

Additions	O_2 uptake (μ atoms)	P esterified (μ moles)	P/O (μ moles P esterified/μ atom O_2 used)
Succinate 0·01M	4·7	8·5	1·8
Succinate 0·01M+ *N. muris* extract	4·7	4·5	0·96
Succinate 0·01M+ EDTA 0·001M	3·8	7·0	1·8
Succinate 0·01M+ *N. muris* extract+ EDTA 0·001M	3·8	8·0	2·1
L-glutamate 0·01M	4·4	10·5	2·4
L-glutamate 0·01M+ *N. muris* extract	4·4	6·5	1·5
L-glutamate 0·01M+ EDTA 0·001M	5·4	16·5	3·1
L-glutamate 0·01M+ EDTA 0·001M+ *N. muris* extract	5·2	16·0	3·1

System: as in Methods; *N. muris* extract prepared as in text, 0·2 ml./flask; duration of experiment, 30 minutes.

Jejunal mitochondria from infested rats prepared and suspended in 0·25M sucrose + 0·003M EDTA oxidize both citrate and succinate at normal rates and are as efficient as control preparations in performing oxidative phosphorylation (Table 7).

TABLE 7.

Effect of EDTA on oxidations and oxidative phosphorylation by jejunal mitochondria.

Additions	$Q_{O_2}^{(N)}$ (μl. O_2 uptake/mg. enzyme N/hour)		P/O (μ moles P esterified/ μ atom O_2 used)	
	Control	Infested	Control	Infested
Citrate 0·01M	110	112	2·0	2·0
Succinate 0·01M	118	110	1·7	1·9

System: as in Methods; enzyme prepared and added in 0·25M sucrose+0·003M EDTA, about 0·7 mg. N/flask; final concentration EDTA, 0·001M; duration of experiment, 30 minutes. Results expressed are the means of two experiments.

Calcium content.

Enzyme and adult parasite preparations were analysed for calcium. Table 8 shows clearly that the uncoupling agent in each case was Ca^{++}, which severely inhibits oxidative phosphorylation in concentrations of 10^{-4}M and greater (Potter, 1947).

TABLE 8.

Calcium content of enzyme and parasite preparations.

Preparation	Experiment	Calcium concentration	
		Mg./100 ml. preparation	Final concentration in reaction mixture
Jejunal mitochondria control—experiment 1	Table 3	0·72	$6·00\times10^{-3}$M
Jejunal mitochondria control—experiment 2	Table 3	0·62	$6·50\times10^{-3}$M
Jejunal mitochondria infested—experiment 1	Table 3	2·58	$2·15\times10^{-4}$M
Jejunal mitochondria infested—experiment 2	Table 3	2·85	$2·38\times10^{-4}$M
N. muris homogenate	Table 4	17·1	$5·70\times10^{-4}$M
N. muris homogenate	Table 5	16·8	$5·58\times10^{-4}$M
N. muris extract	Table 5	12·8	$4·28\times10^{-4}$M
N. muris extract	Table 6	16·0	$5·33\times10^{-4}$M

In the experiments reported in Table 3, the enzyme preparations from infested rats supplied Ca^{++} to give a final concentration in the reaction mixture of 2×10^{-4}M. This concentration of Ca^{++} explains the stimulation of oxida-

tion and inhibition of phosphorylation (Potter *et al.*, 1953; Lindberg and Ernster, 1954). Similarly, worm homogenates used in the experiments of Tables 4 and 5 contained enough Ca^{++} to give a final concentration of 5×10^{-4}M, explaining the failure of phosphorylation, the stimulation of succinate oxidation but inhibition of the oxidation of citrate (Potter, 1947; Potter *et al.*, 1953; Ernster *et al.*, 1955). An intermediate concentration of Ca^{++}, 4×10^{-4}M, supplied by the *N. muris* extract of Table 5 stimulated succinate oxidation, did not alter L-glutamate oxidation and inhibited citrate oxidation but inhibited phosphorus esterification with all substrates. Citrate, which complexes with Ca^{++}, might reasonably be expected to have its oxidation inhibited at a lower Ca^{++} concentration than L-glutamate. Hartman and Kalnitsky (1950) have shown citrate oxidation by liver enzymes to be extremely susceptible to inhibition by Ca^{++}.

Histochemistry.

Calcium could not be demonstrated histochemically in the jejunal epithelium of infested rats. However, large amounts of calcium were found to be accumulated in cells of the parasite gut wall (Figs. 1 and 2).

DISCUSSION.

Host metabolism in tissues other than at the site of infestation might be deranged if parasites were to produce absorbable toxic substances or if they were to deprive the host of certain specific nutrients, for example, vitamins. Materials, other than neutral fats, absorbed from the gastro-intestinal tract pass first through the liver. The presence of toxic substances in the portal blood might well be expected to be reflected in the metabolism of this tissue. Also, the liver is very active metabolically and should quickly show the effects of nutritional deficiencies (cf. Gallagher, Judah and Rees, 1956b). For these reasons liver metabolism in infested rats was studied. It was found to be normal within the limits of the reactions investigated which included the major pathways of intermediary metabolism. Therefore it is concluded as unlikely that the pathological signs of *N. muris* infestation are due either to the absorption of toxic materials or to induced nutritional deficiencies.

Study of jejunal metabolism revealed what appeared to be a most significant biochemical lesion: the uncoupling of oxidative phosphorylation in mitochondria from infested rats.

Many *N. muris* were incorporated inadvertently into the enzyme preparations from infested jejuna. It seemed likely that the inhibitory factor might be added by these homogenized worms. Homogenates of the parasite were, in fact, found to contain a water-soluble substance which uncoupled oxidative phosphorylation in normal liver mitochondria in a manner analagous to that occurring in mitochondria from infested intestines.

The uncoupling agent was in all cases shown to be calcium. Addition of

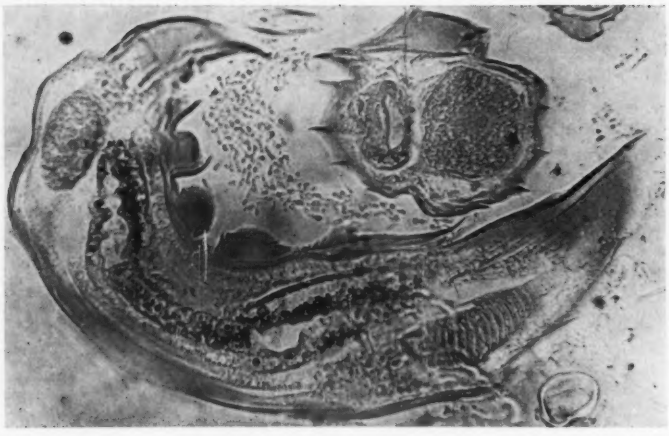

Fig. 1. Longitudinal and transverse section of *Nippostrongylus muris* in an infested jejunum showing accumulation of calcium in cells lining the parasite gut. Calcium is stained by the alizarin red method of Dahl (1952).

Fig. 2. Transverse sections of *Nippostrongylus muris* in an infested jejunum showing accumulation of calcium in cells lining the parasite gut.

ethylenediaminetetracetic acid which chelates Ca^{++} strongly, restores to normal the altered jejunal and liver metabolism.

It is thought to be rather unlikely that calcium derived from the parasite affects host metabolism *in vivo*. However, it is possible that calcium is accumulated in the jejunal epithelium in concentrations sufficient to lower the efficiency of oxidative phosphorylation or to stimulate adenosine triphosphatase activity. Mitochondria have an affinity for calcium. Slater and Cleland (1953) demonstrated that muscle sarcosomes take up large amounts of calcium from solution and Gallagher, Gupta, Judah and Rees (1956) found the calcium content of liver mitochondria to be very greatly increased in rats poisoned with the hepatotoxin, thioacetamide. Jejunal epithelial mitochondria might then accumulate calcium to the detriment of their own biochemical integrity. The answer to this problem must await the isolation of enzyme preparations from infested jejuna without the incorporation of parasites.

Histochemical techniques failed to show any accumulation of calcium in the epithelium but this does not exclude the possibility that inhibitory concentrations might be present. Large amounts of calcium were found to be present in cells lining the parasite gut, undoubtedly the source of calcium concerned in this study. Von Brand (1952) comments on the rather frequent reports of inorganic deposits in the intestinal cells of many nematodes. Such deposits were shown by Quack (1913) and Chitwood and Chitwood (1938) to give reactions characteristic of calcium sulphate. The question arises as to what influence calcium released from the intestinal cells of nematodes by homogenization might have had on studies on the intermediary metabolism of worms.

The study has shown that the severe, acute pathological signs of *N. muris* infestation in the rat are unlikely to result from interference with intermediary metabolism in the liver or in the jejunum. The possibilities that the parasite induces specific nutrient deficiencies in the host or produces substances toxic to the host metabolism are thus argued against. Greater emphasis is now thrown upon the physiologico-pathological aspects of the host-parasite relationship, the possibilities of interference with intestinal motility and the broader mechanisms of absorption.

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